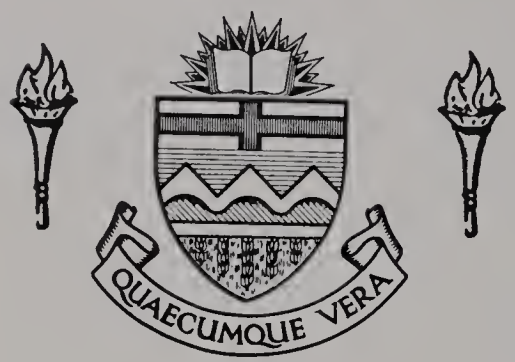

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Genic variation of trembling aspen in Alberta

by

 William Michael Cheliak

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH

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FACULTY OF GRADUATE STUDIES AND RESEARCH

The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research, for acceptance, a thesis entitled *Genic variation of trembling aspen in Alberta* submitted by William Michael Cheliak in partial fulfilment of the requirements for the degree of MASTER OF SCIENCE in FOREST SCIENCE.

DEDICATION

To Leanne,

for her support and encouragement

Abstract

Two hundred fifty-six trembling aspen clones in eight natural trembling aspen populations in Alberta were studied to deliniate variability in leaf morphology. Horizontal starch-gel electrophoresis was used to study crude enzyme extracts from dormant vegetative bud tissue from 222 clones in seven of these eight populations studied for morphology.

The eight morphological characters and two derived ratio characters showed significant ($p < 0.05$) among-population variation. A trend, from south to north, of smaller leaves, with more acute leaf base and vein angles, and more teeth per leaf side was indicated by correlation and discriminant analyses of leaf morphology. Discriminant analysis showed groupings of populations at similar latitudes. The best character for discriminating among-populations was leaf blade length.

A total of 76 alleles coding for 26 putative gene loci were found in the populations studied. Average observed population heterozygosity was 0.52 with 2.3 alleles per locus and 84% of the loci polymorphic. A model for a finite population with neutral alleles was developed to investigate the effects of partial vegetative reproduction on the amount of variation in a population. Under these conditions, the model indicated, vegetative reproduction could have an effect on the amount of variation maintained in the population. However, we are presently unable to determine the magnitude of that effect. Therefore, the high level of

observed variation could possibly be an artifact of the mode of natural reproduction. Within-population variation accounted for 94% of the total gene diversity. Genetic distances among populations were similar to those reported in other studies of woody plant populations. Eighteen percent more individuals were classified into their original poplations by discriminant analysis of genetic data than were classified by discriminant analysis of morphology. There were three significant ($p < 0.05$) canonical correlation functions between leaf morphology and the genetic data.

ACKNOWLEDGEMENTS

I wish to thank the members of my program committee for their comments and critiques of the various aspects of this study. I would also like to give a special thanks to Dr. Ken Morgan for many fruitful hours of discussion, on this study and in general. Thanks to Barry Jaquish for help with the field work.

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I. INTRODUCTION

A. Autecology of trembling aspen

Taxonomy and distribution

Trembling aspen (*Populus tremuloides* Michx.) is one of only two species of Leuce poplars indigenous to North America (Figure 1). It has the widest distribution of any North American woody plant species, spanning 110 degrees of longitude, 47 degrees of latitude and 3000 meters in altitude (Figure 2; Fowells 1965, Harlow and Harrar 1968).

Modes of reproduction

Trembling aspen's primary mode of reproduction in nature is vegetative, but it still retains its sexual capability. The requirements for establishment by each type of reproduction will be discussed to set the framework for a more theoretical discussion to follow.

Sexual reproduction

Trembling aspen is a dioecious species with obligate outcrossing. While hermaphroditism or bisexuality within individual infloresences has been documented (Lester 1963), it is of minor importance in the population. The fertility of seed produced in this manner is unknown.

Trembling aspen flowers and sets viable seed annually, with heavy seed production occuring every 4 to 5 years

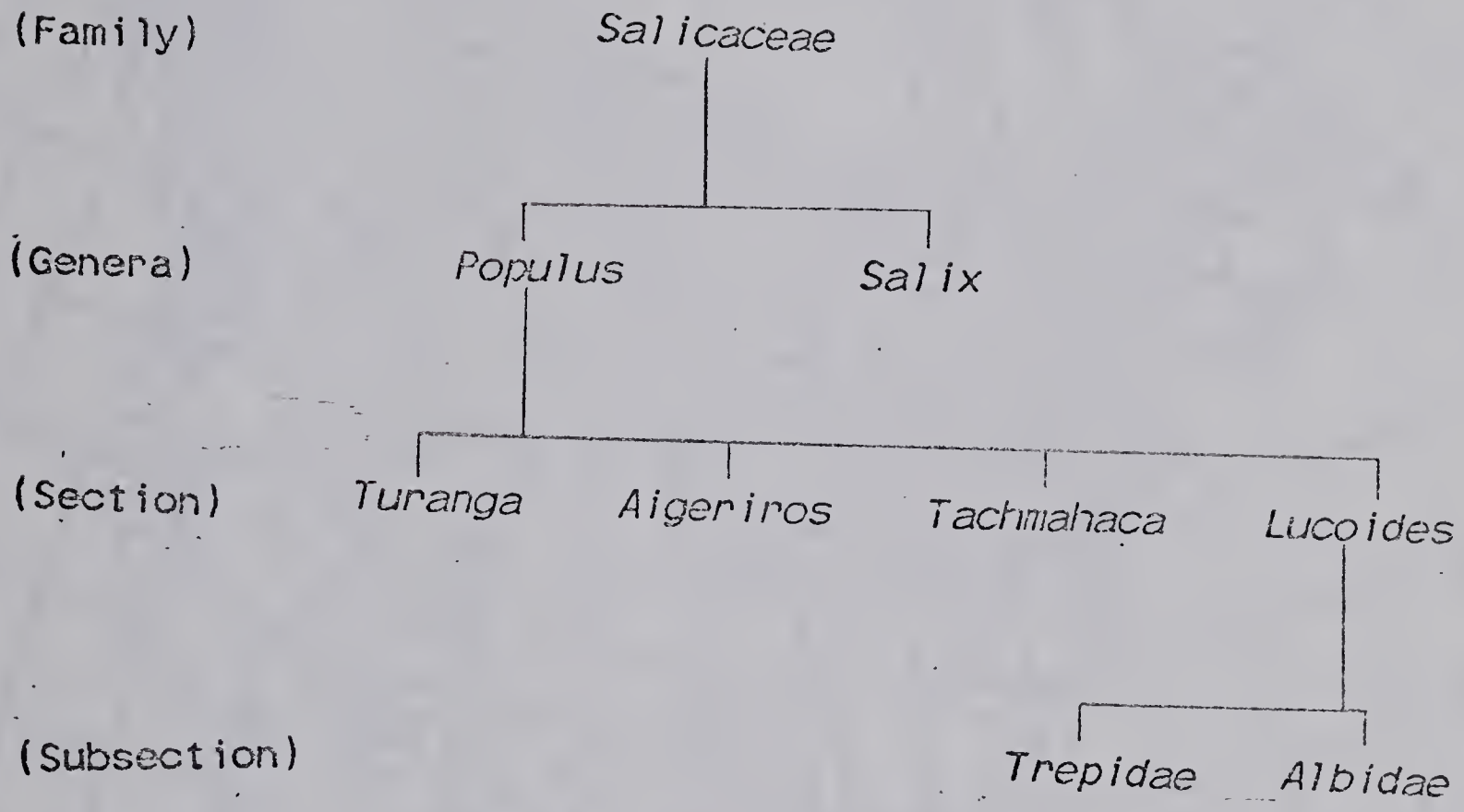

Figure 1. The taxonomy of the *Salicaceae* family (Annon. 1965).

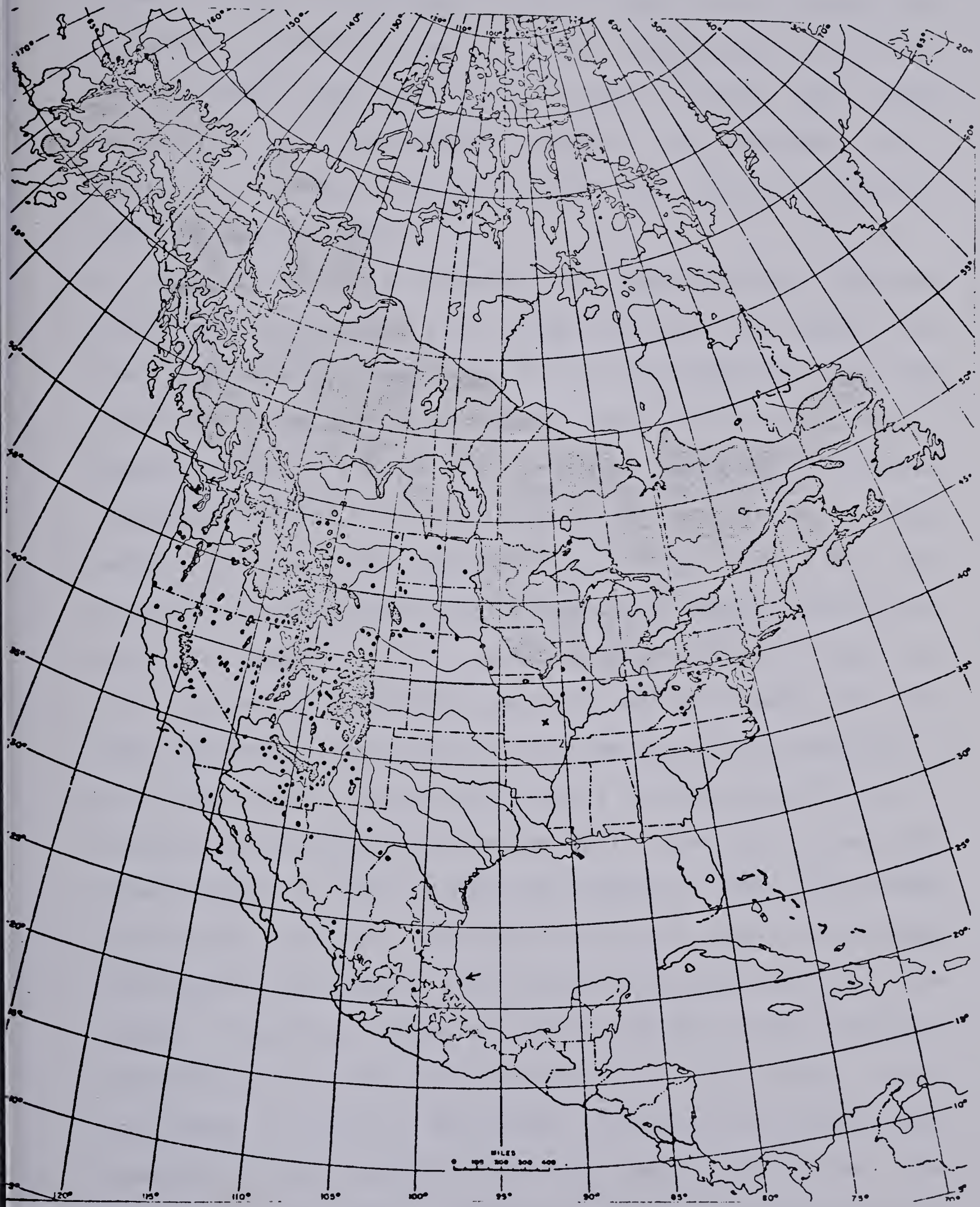

Figure 2. The botanical range of *Populus tremuloides* Michx. (Fowells 1965)

(Fowells 1965). An average - sized female can produce an estimated 1,625,000 seeds in an average year (Maini and Cayford 1968). With the amount of seed produced, one would expect to find substantial numbers of seedlings in a population. However, several factors act strongly against seedling establishment.

Of primary importance are the exacting moisture conditions required to initiate germination and carry the newly germinated seedling through a prolonged delicate and succulent three-week stage. Aspen seeds are extremely small, (approximately 2.6 million cleaned seeds.Kg^{-1}, Cheliak unpublished data), and, therefore, do not have a large amount of stored carbohydrates. Thus, the tiny seed generally germinates on the soil surface, where inconsistent moisture regimes exist, and begins producing its own food for root system growth and seedling establishment. If the new germinant dries out at any time during this period, it will die. Another factor influencing seedling survival is the extreme intolerance to shade of the species. My personal observations of aspen seedlings growing under greenhouse conditions indicate that over-topping of even 2 cm between two adjacent seedlings is sufficient to cause death of the shorter seedling. These are the two major factors limiting establishment by seed, but other factors (e.g., frost) could contribute to early mortality. Considering the problems associated with establishment by seed, it is not too surprising that the predominant mode of reproduction is

vegetative. Despite the above mentioned difficulties, however, seedlings do become established, though, apparently infrequently.

Andrejak and Barnes (1969) reported a seedling population of aspens (*Populus tremuloides* and *Populus grandidentata* Michx. and their hybrid) in southeastern Michigan. Of 78 stems in the population, 54 (69%) were definitely seedlings (35 stems (65%) *P. grandidentata*, 14 stems (26%) *P. tremuloides* and 5 stems (5%) putative F1 hybrids). However, the conditions in the area of establishment were rather unique. The stand was located on an abandoned agricultural field, and weather records showed both temperature and precipitation at the time of germination and for approximately a month after were above average.

Faust (1936) and Larson (1944) both reported the occurrence of trembling aspen seedlings along the shorelines of receded reservoirs. Ahlegren (1959), Barnes (1966), Gauvin (1964), MacArthur (1964), Mills (1961) and Vogl (1969) reported finding aspen seedlings on recently burned sites. Ellison (1943) reported finding an aspen seedling on a moist, high elevation site in southern Utah. Graham *et al.* (1963) reported finding aspen seedlings in the seedbeds of a forest nursery.

Thus, given present day conditions, seedlings can occasionally become established in a population. The frequency with which seedlings are reported in the present

literature is probably biased downward due to the difficulty of unambiguously determining seedling identity. Given that trembling aspen can become established by sexual means, what is the probability that a seedling will persist in the population long enough to reproduce? Barnes (1966) reported that even when inital establishment conditions were satisfied, the 18 to 450 newly germinated seedlings were not alive after a period of two years.

From the evidence presented we may conclude that the proportion of the population derived from recent sexual generations is negligible. These sets of observations probably led Baker (1925), Cottam (1954, 1966) and Moss (1938) to propose that trembling aspen had effectively ceased establishment by sexual means in Western North America in the late Pleistocene. While these estimates are not directly testable, they do emphasize that establishment and subsequent survival of seedlings are very difficult. If this were not true, the clonal mode of reproduction would probably not be as ubiquitous.

Asexual reproduction

The asexual or clonal nature of trembling aspen was first demonstrated by Leopold and Jones (1947) and Pauley (1949), although silviculturalists such as Baker (1921) first reported marked differences in spring phenology and bark types of trembling aspen in Utah. Baker considered

these differences to indicate the existance of two races of aspen, while others termed aspen clones, "groves".

The majority of trembling aspen suckers (ramets) arise from the existing root system after apical dominance exerted by the parent stem (ortet) has been broken by some type of disturbance (Farmer 1962, Eliason 1971a, 1971b, 1972, Schier 1972, Steneker 1974). Extensive studies have been undertaken on the origin, physiology and development of aspen suckers by Schier and others (Peterson 1975, Schier 1972, 1973a, 1973b, 1973c, 1974, 1975a, 1976, 1978a , Schier and Johnstone 1971, Schier and Smith 1979, Schier and Zasada 1973, Skene 1975, Williams 1972, Winton 1968a, Wolter 1968, Zasada and Schier 1973). These investigations showed:

(i) suckers originate from newly formed meristems, pre-existing primordia or suppressed short shoots;

(ii) auxin concentration influences sucker production in a negative way;

(iii) concentrations of cytokinin, gibberellin, kinetin and *B*-inhibitor positively influence sucker production;

(iv) temperature has a positive effect on sucker production; and

(v) there are large clonal differences in ability to produce suckers.

Since auxin is synthesized in swelling buds, growing shoots and leaves (Eliasson 1971a), the role of disturbances which cause death of the above-ground portion of the tree, such as fire, harvesting or avalanches, is of primary

importance in the maintenance of aspen forests (Barnes 1966, Graham *et al.* 1963, Loope and Grunell 1973, Marr 1961). Schier (1975b) proposed that some aspen clones deteriorate as a function of the slow death of the mature stems over time. He suggested that auxin is being produced in sufficient quantities by the remaining living stems in a clone to supress any significant sucker production. Additionally, the shade from the stems left standing can significantly reduce sucker growth or, in time, eliminate all suckers that have originated during the slow disintegration of a clone. However, even a very scattered few individuals or root-stocks are sufficient to reclaim an area once another disturbance occurs (Schier and Smith 1979).

With the information given, a clone could be expected to develop as follows. Assume that at some point in time a seedling has become established in a population. When it is destroyed, apical dominance will have been broken, enabling suckers to arise from the shallow lateral roots of the original root system. Over time, the clone, which represents a particular genotype, can either expand, disappear or remain static, depending upon its fitness. Ignoring chance historical events such as agriculture or road building activities and considering the case of clonal expansion, the vegetative increase can theoretically continue without limit. Practically, however, this expansion is limited by the availability of suitable sites and competition with

peripheral individuals (or other clones). This situation precisely fits the Strawberry-Coral model of Williams (1975).

In trembling aspen, the largest reported clone consists of some 47,000 stems occupying an area of 43.3 ha in Fish Lake Basin, Sevier County, Utah (Kemperman and Barnes 1976). In recently-glaciated areas, the largest aspen clone reported occupies an area of 1.5 ha in Riding Mountain National Park, Manitoba (Steneker 1973). As part of an aspen study by the Department of Forest Science, University of Alberta, the largest clone mapped to date occupies 0.95 ha, and is located northwest of Edmonton, Alberta (Jaquish pers. comm.).

B. Variability

Virtually every characteristic of trembling aspen studied has shown significant inter-clonal phenotypic and/or genetic variation. Some of the more important characters from a population point-of-view are discussed below.

Unlike most species, the basic sampling unit in trembling aspen is the clone (Zahner and Crawford 1965). Therefore, it is imperative that one be able to unambiguously identify individual clones in the field. Failure to identify clones would result in an underestimation of the the population variability due to multiple samplings of the same genotype. Traditional approaches to the problem of clonal identification in aspen,

involve using a combination of characters (Barnes 1969; Table 1) largely dependent upon season.

In additon to simple clonal identification, many of these characters also have been used to assess phenotypic variation in aspen (Andrejack and Barnes 1969, Barnes 1969, 1975, Farmer and Barnes 1978, van Buijtenen *et al.* 1959). In a large study by Barnes (1975), several leaf characteristics showed north-south, east-west and altitudinal clines of variation. In addition, the patterns of character variation very closely corresponded to patterns of environmental variation (e.g. large leaves measured on trees in moist environments).

Covington (1975) reported an altitudinal cline of chlorophyll concentration and reflectence of the bark of aspen in New Mexico. Since aspen bark contributes some 15 percent to total photosynthetic area, (Schaedle *et al.* 1968) and this portion is fully capable of photosynthesizing below ambient temperatures of 0° C (Pearson and Lawrence 1958), it has been suggested that this is one attribute of trembling aspen that might account for its extreme latitudinal and elevational distribution (Strain and Johnstone 1963). These results showed a significant negative correlation between altitude and bark chlorophyll concentration. Since bark reflectence showed a positive relationship with altitude, however, photosynthetic capabilities probably are similar at different elevations.

One of the most conspicuous among-clone differences in

Table 1. Charcteristics for field identification of trembling aspen clones ranked by season in decreasing order of usefulness (from Barnes 1969).

A. SPRING
   1. Sex
   2. Time of flowering and floral characteristics
   3. Time, color, and progression of leaf flushing

B. AUTUMN
   4. Leaf coloration
   5. Time and progression of leaf fall

C. SUMMER
   6. Leaf shape (BW/BL ratio) color and size
   7. Configuration of blade base
   8. Leaf margin: tooth number, size and shape
   9. Configuration of blade tip

D. ALL SEASONS

Bark Characteristics

   10. Bark texture
   11. Bark color

Stem Characteristics

   12. Stem form
   13. Branching habit (branch angle, branch length, internode length)
   14. Susceptibility to injury
      a. Sun-scald
      b. Frost crack
      c. Insect and disease injury

Others

   15. Miscellaneous characteristics
      a. Pruning ability
      b. Leaf rust
      c. Aphid galls
   16. Vertical clone profile

aspen is the timing of various phenological events such as leaf flush or autumnal coloration and senescence. It is not uncommon to have a three-to four-week difference between leaf flush of the earliest and latest clones in an area (Baker 1921, Barnes 1969, Cottam 1954, Covington 1975, Egeberg 1963, Kemperman 1977, Strain 1966). Within a single

clone, there is some variablility in flushing, but this variation is a function of the microclimatic or environmental differences within the clone (e.g. first flushing in the upper crown by the largest ramets in the southwest quadrant; Barnes 1969).

While early flushing may afford the particular clone an advantage in growth (Strain 1966), it also increases the probability that it will be damaged by late spring frosts (Egeberg 1963). Zalasky (1976) reported two main types of frost damage to poplars;

(i) outright killing (canker or dieback) and

(ii) distortion of developing organ and plant parts in the area surrounding the canker or dieback.

The necrotization of tissues with subsequent rupturing of the bark provides suitable infection courts for a variety of pathogens (Smith 1970).

Clonal variation in resistance of aspen to various pathogens has been documented by several authors (Copony and Barnes 1974, French and Hart 1978, French and Manion 1975, Schipper 1975, Valentine *et al* 1975, and Wall 1971). The most intensively studied pathogen attacking aspen is *Hypoxylon mammatum* (Wahl.) Mill. The main results of these studies showed that there was non-significant intra-clonal variation but highly significant inter-clonal variation in susceptibility to *Hypoxylon* canker. There is good evidence that this inter-clonal variation is not an artifact of environmental variation, but is under polygenic control

(Manion and Valentine 1974, Valentine and Manion 1972 and Valentine *et al.* 1975). Valentine *et al.* also presented evidence that this resistance mechanism is in the form of a hypersensitive reaction by the host (i.e., callous tissue formation). This trait exhibited Mendelian segregation patterns.

Schipper (1975) and Wolter and Gorden (1975) reported changes in aspen peroxidase isoenzyme profiles in reaction to tissue innoculated with *Hypoxylon*. Peroxidase seems to be a good indicator of host resistance, in light of evidence presented by Beckman (1979) implicating peroxidase as an essential agent in the hypersensitive reaction of plants to resist infection by pathogens.

The ability of an organism to reproduce and thereby have its genes represented in ensuing generations is of paramount importance in determining its fitness. In trembling aspen, variation in ability to reproduce varies not only with season and other environmental parameters, but also, most importantly, with clones.

Soil temperature, whether increased from burning or from increased solar radiation reaching the ground surface, promotes suckering in aspen (Maini and Horton 1966 Schier 1976). Horton and Hopkins (1965) found that suckers arising from "moderate" post-logging burns had significantly better height growth at the end of the first growing season (0.8 m verses 1.0 m). Schier and Campbell (1978) found that post-logging burning increased the depth at which suckers

were initiated, thereby increasing the overall density of the newly arising stands. Zasada and Schier (1973), working with roots collected from three clones suckered in a common environment found that, the number and dry weight of suckers produced increased with temperature (up to the maximum of 30° C day / 20° C night). However, they found, as did Maini (1967), that there were clone (genotype) x temperature (environment) interactions in the development of aspen suckers. Thus, the importance of fire in the maintenance of aspen populations stems not only from the breaking of apical dominance to initially stimulate suckering, but also in increasing the numbers and vigor of the suckers produced.

While fire may stimulate sucker initiation from a greater depth within the soil, these newly initiated suckers are most likely to be totally dependent upon food reserves (primarily carbohydrates) in the parent root system until they reach above the soil surface and start photosynthesis on their own (Schier 1976, Schier and Zasada 1973).

Work by Schier and Johnstone (1971) and Schier and Zasada (1973) showed that:

(i) there are large clonal differences in levels of total non-structural carbohydrate (T.N.C.) reserves;

(ii) these differences, infered from individual sucker dry weight, are manifested to a greater degree when suckers are propagated in the dark (i.e., simulating soil conditions); and

(iii) there are drastic seasonal changes in T.N.C.

concentration directly related to phenological events.
The previous examples show how environment (temperature and T.N.C. reserves) affects the suckering (reproductive) ability of a clone. More important for this study, however, is the fact that the clones or genotypes also show significant variability in their ability to sucker.

Assessing genetic variation

To directly assess genetic variation, one can assay enzymes through the technique of electrophoresis. Since enzymes, which are proteins, are the first metabolic product from the transcription of a gene, they can be related directly to the allelic constitution of a particular individual (e.g., Conkle 1971, Feret 1972, Mitton *et al.* 1977, Miyasaki and Sakai 1969 and Snyder and Hamaker 1978). Harris (1966) and Lewontin and Hubby (1966) were the first to apply electrophoretic surveys of genic variation to the study of populations. Since these initial applications, the number of investigations employing some type of electrophoretic technique has expanded rapidly. In general, most studies show an excess of electrophoretically detectable variation over what would be predicted by the classical hypothesis of population structure. Since, electrophoretic identity requires a mutation to result in a change in net electric charge, electrophoresis is theoretically capable of detecting between 25 and 35 percent

of the total genetic variation in the population (King 1973, Lewontin 1974, Nei and Chakraborty 1973). A controversy now exists between two schools of thought, the selectionists and the neo-classicists or neutralists, regarding the significance of this variation.

The neo-classicist would account for this excess of variation by claiming that it is apparent and not real. That is, although detectable electrophoretically, in most cases it is not detectable by the organism. Therefore, these alternate allozymes (allelic isoenzymes (Scandallios 1969)) are "genetic junk" and redundant physiologically (Lewontin 1974). Proponents of this hypothesis suggest three classes of mutations giving rise to electrophoretically observable variation. The first class is subject to natural selection, but these mutations are almost all deleterious and would be removed from the population. The second class includes redundant or neutral mutations, which are not subject to natural selection. Typically, these are the alleles observed segregating in electrophoretic profiles. The last class is the rare favorable mutation, which will become fixed by natural selection and form the basis for adaptive evolution.

The selectionists or balance school maintain that these various electromorphs (King and Ohta 1975) do differ in fitness (i.e., they confer some sort of adaptive superiority) and that they are maintained in populations by various forms of balancing selection and heterosis.

Because of the robustness of each theory, almost any

set of observations can by explained from either positon. This fact, coupled with the lack of statistical power associated with any present test (e.g. Lewontin and Kraukauer 1973) for these hypothesis, makes it unlikely that either hypothesis will be refuted in the near future.

C. Purpose and objectives

Despite these problems, electrophoresis still remains the most powerful tool that the experimental population geneticist can employ. Presently, it is the only method which allows rapid screening of an unbias sample of the genome of a large number of individuals. This technique allows the experimentalist to survey genetic variation at the level of an allele, something theoreticians have been pursuing since the 1930's. These surveys of allelic or genic variation can then be used not only to describe the genetic structure of populations and the amounts of variability maintained in populations, but also, can serve as the basis for testing predictions from theoretical models.

Several unique features of trembling aspen, such as its extreme distribution limits, great phenotypic variability and mode of natural reproduction, provide an interesting background for studies on population genetics. To date, the majority of variability studies at the population level in trembling aspen have studied phenotypic characters. The primary purpose of this study was to document the genetic variability and genetic structure of trembling aspen

populations in Alberta. Specifically, the objectives of this study were:

(i) to describe the variability in characters of leaf morphology, traditionally used in studies of trembling aspen, sampled in Alberta;

(ii) to describe the genetic structure of trembling aspen populations in Alberta; and

(iii) to determine whether there are any relationships between genetics and morphology in these populations.

II. MATERIALS AND METHODS

A. Collections

Sampling locations

Eight locations in southern and central Alberta were sampled on the premise that since they were geographically distant, they were presumably ecologically different (Figure 3). At each sampling location, a minimum of 30 ramets, each representing a different clone (delineated according to the characters specified in Table 1 and spatial isolation), were chosen to represent that particular population (see Appendix 1 for sample size justification). These ramets were marked with flagging tape and numbered with aluminum tags to facilitate subsequent collections.

Leaf collections and measurements

Five short shoots 2-6 cm long were collected from the lowest branch on the south-western exposure of each ramet. This standardized approach to collection tends to maximize the probability of uncovering "genetic based variation" (Barnes 1975) by minimizing confounding extraneous intra-clonal or error variation (Dancik and Barnes 1974). The middle leaf from each short shoot was chosen for all subsequent measurements.

Eight measurements were made (Figure 4) on five leaves from each ramet and averaged for final analyses. This

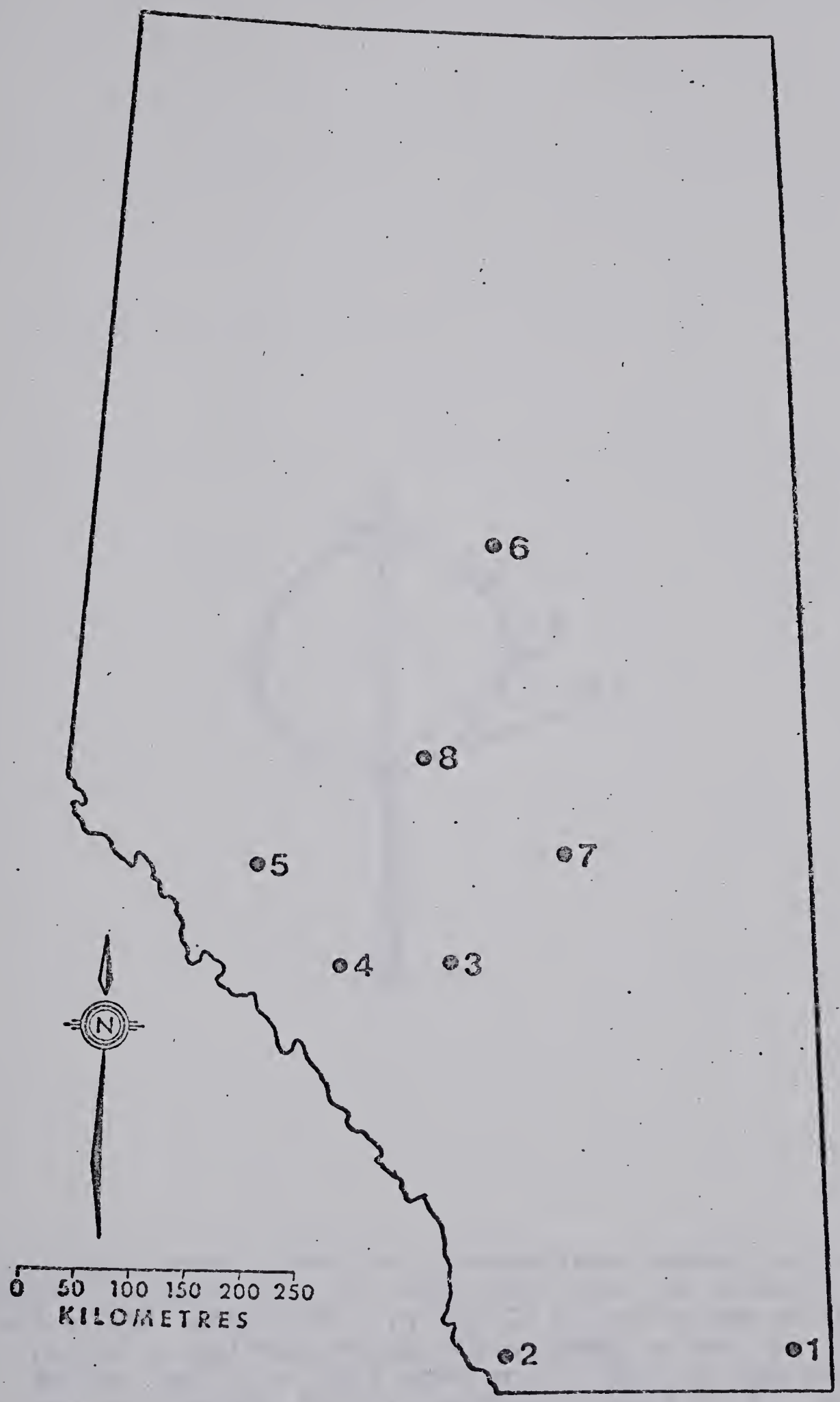

Figure 3. Outline map of Alberta showing the location of the eight natural trembling aspen populations sampled.

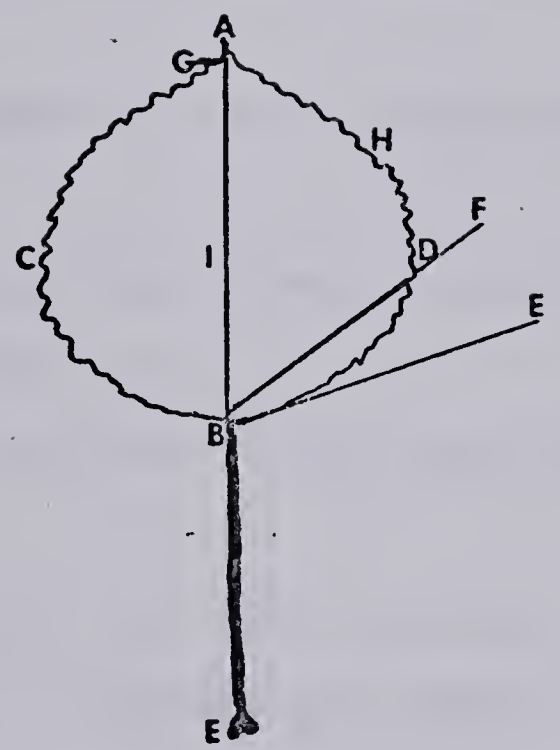

Figure 4. Measurements made on standardized leaves collected from seven natural populations of trembling aspen in Alberta.
AB = leaf blade length (BL) in mm; CD = leaf blade width (BW) in mm; BI = point of maximum blade width (MBW) in mm; BE = petiole length (PL)in mm; AE = leaf base angle (BA) in degrees from A = 0; AF = leaf vein angle (VA) in degrees from A = 0; AG = apex length (AL) in mm; H = number of teeth on one side of the leaf blade (NOTETH); In addition two ratios were calculated: BW/BL, MBW/BL.

averaging opens up the possibility of introducing an "errors in variables" problem (Madulla 1977). In addition to the errors-in-variables (or measurement error), an error covariance was introduced from the heterogeneity in physical leaf size. The general problem can be stated as follows;

$x(ij) = u(ij) + e(ij)$, where;

$x(ij)$ is the estimated mean value of trait i measured on individual j,

$u(ij)$ is the true mean of trait i measured on individual j and

$e(ij)$ is the measurement error associated with trait i measured on individual j.

A more technical treatment of this problem can be found in Appendix 2.

The net effect of this problem is to reduce the correlations (or in regression models, to decrease the regression coefficient and the multiple correlation coefficient; Cochran 1970, Warren *et al.* 1974) among the variables in the data set. Since the starting point for the multivariate techniques used in this study is a variance-covariance matrix or a correlation matrix, it was felt that this measurement error and size heterogeneity problem could introduce bias in the results. If the magnitudes of measurement error were the same across the variables measured, the eigenvalues and elements in the

eigenvectors would be reduced by a scalar. This would result in reduced explanitory power of the technique due to random errors. However, different aspects of leaf morphology were considered, each with a different precision. Therefore, different magnitudes of measurement error were expected.

This problem was corrected in the discriminant analysis of leaf morphology. The procedure used to correct for the errors-in-variables problem was as follows;

(i) Obtain a variance-covariance matrix which consists of estimates of the variance and covariance associated with the values used to obtain the mean value per individual.

(ii) Since we are estimating the variance of a mean, it is necessary to further reduce these estimates by the number of observations which were used in the estimate (in this problem, 5).

(iii) Obtain an estimate of the pooled within-groups variance-covariance matrix.

(iv) Obtain an estimate of the corrected pooled within-groups variance-covariance matrix by subtracting the result of (ii) from (iii).

All parameters measured correspond to those traditionally used to establish patterns of phenotypic variation in trembling aspen (Barnes 1966,1969,1975, van Buitjinen *et al.* 1959 and Farmer and Barnes 1978).

Tissue collection

Initially, root sections were collected from each selected clone in the spring of 1979 and placed on trays, under mist in the greenhouse, with conditions optimal for sucker growth (Schier 1978b). The resultant suckers were frozen (in whole or block form) in liquid nitrogen for 5 seconds and kept in a chest freezer (-20° C) until used for electrophoresis, (Brewer 1970).

This material provided inconsistent results. Often, complete enzyme systems were destroyed. Occasionally, a few individuals would show reduced activity for one enzyme system, but resolution was poor. The only enzyme which was consistently active and had good resolution with this material was peroxidase. Using the sucker tissue, different peroxidase loci were expressed in the various organs (e.g., leaves, petioles and stems). In an attempt to alleviate these problems, dormant vegetative bud material was used. Since it was necessary to re-collect the samples in the field, problems of accessibility limited the number of samples for which there were both morphological and genetic data to 196. Most of this loss was due to the elimination of the Whitecourt population (number 8).

The complete data sets are those which will be used for the separate morphological and genetic analyses. Partial or reduced data sets are those which have both morphological and genetic data in common.

B. Electrophoresis

Tissue preparation

Between 80 and 100 mg of tissue (with bud scales intact) was cut into small pieces and placed in a 2.0 ml pyrex borcelite glass homogenizing tube with 8 drops (ca. 100-130 *ul*) of ice-cooled (ca. 0° C) extraction buffer modified from Coulthart (1979). After hand homogenization, the homogenate was absorbed onto three 4 x 10 mm (Wattmann No. 3 qualitative) paper wicks. The wicks were placed sequentially on a microscope slide in a covered petri dish with a distilled water-soaked filter paper to provide increased humidity, thereby reducing homogenate evaporation. The petri dishes were placed on packs of ice to keep the temperature between 0 and 2° C. The homogenates were stored in this manner for up to 3 hours (while homogenization was taking place) with no apparent degradation of the enzyme systems studied. At no point in the extraction process did the temperature of the homogenate exceed 4° C.

Electrophoresis

Starch gels (12.5%) were prepared acccording to the general method of Smith (1976) with hydrolized potato starch (Electrostarch Co., Madison, Wisconsin). After heating and degassing, starch was poured into a 350 ml (14.5 x 9 x 22 cm) plexiglass mold. After solidifying, the gels were covered with Saran Wrap to prevent dessication. Gels were

cooled for two to three hours to room temperature while homogenization of the bud material was taking place. Personal experience indicated that if the gels were cooled to below room temperature, the buffer would not break through the origin evenly. This made scoring the gels difficult or impossible.

Two different buffer systems were used to satisfactorily resolve the 13 enzyme systems studied (Table 2). For both buffer systems, the wicks were left intact for the entire run. For each buffer system the sample was introduced into the gels at a potential of 100 volts (V) for one-half hour.

Electrophoresis was conducted for 3.5 hours at 250 V on the Tris-Versane-Borate (TVB) system. At this time, the enzymes had migrated approximately 6 cm. Since the Lithium-Citrate (LC) system has an interactive marker front, migration was allowed to proceed for 8 cm (taking approximately 4 hours) at a potential of 250 V.

Two gels were run off the same LC buffer tanks by vertically stacking the two gels and doubling the electrode wicks leading to the top gel. This resulted in approximately equal rates of migration for both gels. After the inital sample introduction into the medium, ice packs were placed on top of each gel; gel temperature was thus maintained at about 1° C.

After electrophoresis, the gels were sliced with 0.007 mm nylon monofilament line into 1 mm slices. The top and

Table 2. Enzymes assayed, running buffers and number of loci scored in a study of seven natural populations of trembling aspen from Alberta.

ENZYME SYSTEM	ABBREVIATION	BUFFER SYSTEM	NUMBER OF LOCI SCORED
Aspartate aminotransferase	AAT	A	2
Alkaline phosphatase	ALP	A	1
Acid phosphatase	APH	A	3
Catalase	CAT	B	1
Colorometric esterase	CLE	B	3
Floroumetric esterase	FLE	B	3
B-Glucosidase	*B*-GLD	B	1
Hexosaminidase	HEA	B	1
Leucine aminopeptidase	LAP	A/B	3
Peroxidase	PER	A	3
Phosphoglucose isomerase	PGI	A	3
Phosphoglucomutase	PGM	A	1
Superoxide dismutase	SOD	A	1

Buffer systems	**Reference**
A=Tris/Citrate:Lithium/Borate pH 8.5/8.1	Ridgeway *et al* 1970
B=Tris:Versane:Borate pH 8.0/8.0	Sicliano and Shaw 1976

bottom slices of each gel were discarded. Each slice of gel was then stained for one particular enzyme.

Enzyme staining recipes (Appendix 3) were modified slightly from those given by Brewbaker *et al.* (1968), Mitton *et al.* (1979), Shaw and Prasad (1970), Siciliano and Shaw (1976) and Yeh and Layton (1979).

Putative gene loci were inferred from patterns of segregation observed in the preliminary screening of the enzyme systems. The fastest anodally migrating locus was desiginated as number 1. The most anodal putative allele at that locus was desiginated A1. Different alleles at a locus were confirmed by measurement relative to a standard. The numbering of alleles and additional loci within an enzyme system progressed sequentially in the cathodal direction. Thus, each enzyme was desiginated by an abbreviation (see Table 2), a number which indicates the putative locus and an A followed by a number which indicates the allele at that locus. For subsequent analyses, these results were recoded according to the method of Smouse and Neel (1977).

C. Analyses

Univariate analyses

One-way analysis of variance of each character in the morphological data set was made to evaluate among-population variation. Multiple comparison tests, (Student Newman Keul; Steel and Torrie 1960) were made to determine those populations which were significantly different from each

other. For the morphological data, Pearson product moment, Kendall and Spearman rank correlation coefficients were computed to determine inter-relationships within and among the various types of variables (e.g., metric, angular and non-metric). Clinal patterns of variation in the morphological data set were examined by Pearson product moment correlations of morphological character population means with latitude, longitude, and elevation.

Descriptive Population Parameters

Expected heterozygosity (E(h)) was calculated for each locus by population to compare interpopulation variablity at a gene locus. The E(h) is calculated as,

$$E(h) = 1 - \sum_{ij} p(ij)^2,$$

where p(ij) is the frequency of the i *th* allele in the j *th* population.

Deviations from Hardy-Weinberg expections using Levene's (1949) modification for small sample size were calculated for each variable locus in each population and tested by a maximum likelihood G^2 (assymptotic X^2) statistic. Under the assumptions of random mating with no selection, mutation, migration or random genetic drift, the observed genotypic proportions are not expected to deviate from the predicted proportions. When there is a deviation (either an excess or deficiency of heterozygotes) one or possibly all of these factors could be operating.

The organization of the genetic variability in trembling aspen was analyzed by Nei's subdivided population statistics (Nei 1973). From the total population gene diversity, H(t),

$1 - \sum j ((\Sigma i\ x(ij)/s)^2)$, where

x(ij) is the frequency of the i *th* allele in the j *th* subpopulation and s is the number of subpopulations, the gene diversity residing within subpopulations and between subpopulations can be partitioned into separate components, H(s),

$1 - \sum i ((\ \Sigma\ j\ x(ij)^2)/s))$ and D(st),

H(t)-H(s) respectively. The relative measure of genetic differentiation between subpopulations is then given by,

G(st),

D(st)/H(t).

To compare the average number of net codon differences per locus between populations, Nei's standard genetic distance (Nei 1972) was used. Genetic distance is defined as,

D (genetic distance) = -log(e)I where

I (gene identity) = $J(xy)/(J(x)J(y))^{-1/2}$ and

$J(xy) = \sum i\ x(i)\ y(i)/l$,

$J(x) = \sum i\ x(i)^2/l$,

$J(y) = \sum i\ y(i)^2/l$,

where x(i) is the frequency of the i *th* allele in population X, y(i) is the frequency of the i *th* allele in population Y and l is the number of loci in the data set. The geographic

distance between populations was measured to determine if there was a relationship between genetic distance and geographic distance. A chi-square analysis of allelic homogeneity was performed on all variable gene loci across the populations.

Multivariate analyses

Discriminant analysis was performed separately on the genetic and morphological data sets using the populations sampled to define group membership. Input data involved using the corrected variance-covariance matrix of morphological traits and the common allele at every variable gene locus. Since most of the information for all the gene loci studied was contained in the most common allele, it was most efficient to include only the one allele at each locus. Discriminant analysis finds the linear combination of the original variables which best separates or discriminates between pre-defined groups. The criterion used for group separation was the maximization of the minimum Mahalanobis D^2 between groups. Canonical correlation was used to determine what, if any, linear transformations existed within the data sets such that the correlations between the morphological and genetic data were maximized. Since canonical correlation can be thought of as a multivariate regression technique, it is useful for determining the predicitive power of one set of data for the other set. The

raw morphological variables and the common allele at every variable locus were used as input variables for the canonical correlation analysis. Only individuals for which morphological and genetic data were available were considered in this analysis.

Computer Programs

All statistical analyses were run through the SPSS (Nie *et al.* 1975) subroutines. The genetic distance program was written by Dr. Ken Morgan of the Genetics Department at the University of Alberta. The author wrote the program for Nei's subdivided populations and the X^2 analysis. A statistically significant difference was declared at alpha = 0.05.

III. RESULTS

A. Morphology

Univariate analyses

Of the morphological characters, leaf apex length had the largest variation among the eight populations sampled (Table 3). Within populations, only the two ratio variables (BW/BL, MBW/BL) were not normally distributed according to the Kolmogorov-Smirnov test of normality (K-S(Z)). However, when the data for all populations were pooled, blade length, base angle and apex length also show non-normal distributions. The above mentioned variable distributions all showed a positive skew (i.e., to the left of the mean) and were more narrow than would be expected from a normal distribution.

There was considerable variation among the eight populations for all measured traits (Table 4). Using a one-way analysis of variance (ANOVA), every variable showed significant variation among populations. Except for number of teeth, the ranking of the various populations remained relatively constant among other variables when subjected to a multiple range test (Student Newman Keul).

Pearson product moment correlations were relatively strong ($r > .7$) and positive among the four metric characters, blade length, blade width, maximum blade width and petiole length (Table 5). Apex length showed moderate ($r = 0.4 - 0.7$) correlations with blade length and maximum

Table 3. Descriptive statistics for leaf morphology based on eight natural aspen populations in Alberta.

	STATISTIC							
VARIABLE*	X	se	s	K	S	K-S(Z)	(P)	CV
BL	35.6	.4	6.4	6.0	1.7	1.518	.02	17.98
BW	34.0	.4	6.1	.1	.2	.725	.67	17.94
MBW	15.1	.2	3.5	5.1	1.6	1.344	.06	23.18
PL	26.3	.4	5.6	.2	.4	1.195	.12	21.29
BA	64.0	.7	10.5	.1	.8	1.774	.00	16.41
VA	40.6	.4	5.8	.0	.2	.886	.44	14.29
AL	3.6	.1	1.4	1.9	1.1	1.815	.00	38.89
NOTETH	22.6	.2	3.6	.1	.5	1.045	.22	15.93
BW/BL	1.0	.0	.1	.2	.3	3.553	.00	10.42
MBW/BL	.4	.0	.1	.1	.3	5.865	.00	14.26

*X = character mean; se = standard error of the character mean; s = character standard deviation; K = Kurtosis; S = Skewness; K-S(Z) = Kolmogrov - Smirnov Z value; P = probability statement for the K-S(Z); CV = coefficient of variation; * = P(<0.05).*
* See Figure 4 for descriptions of the variables and their abbreviations.

Table 4. Morphological character means for eight natural aspen populations from Alberta.

POPULATION	CHARACTER*									
	BL mm	BW mm	MBW mm	PL mm	BA °	VA °	AL mm	NOTETH #	BW/BL	MBW/BL
1	38	37	18	29	74	44	4	20	1.0	.5
2	37	38	17	28	81	46	3	21	1.1	.5
3	42	38	19	29	60	39	4	25	.9	.4
4	33	29	15	24	62	38	3	22	.9	.5
5	35	35	13	26	59	42	3	23	1.0	.4
6	34	32	13	24	55	40	4	23	1.0	.4
7	35	33	15	27	62	39	3	25	1.0	.4
8	33	30	12	22	62	38	3	21	.9	.4

* see Figure 4 for character descriptions and their abbreviations

Table 5. Univariate (Pearson product moment) correlations among the morphological characters measured on eight natural populations fo trembling aspen in Alberta and their geogarphic location.

	BL	BW	MBW	PL	BA	VA	AL	NOTETH	BW/BL	MBW/BL	LAT	LONG	ELEV
BL	1	.8*	.8*	.8*	.2*	.1	.6*	.2*	-.2*	.1	-.4	-.2	.2
BW		1	.7*	.8*	.3*	.3*	.4*	.2*	.4*	.1*	-.6	-.3	.2
MBW			1	.7*	.3*	-.2*	.5*	.1	-.2*	.5*	-.6	-.4	.6
PL				1	.2*	.0	.3*	.2*	.1	.1	-.7	-.3	.4
BA					1	.3*	.1	-.2*	.2	.3	-.9*	-.5	.5
VA						1	.0	-.1	.5*	-.3	-.7	-.2	.3
AL							1	.0	-.3*	.1	-.3	-.6	.1
NOTETH								1	.1	-.2	.6	.6	-.2
BW/BL									1	.0	-.6	-.1	.1
MBW/BL										1	-.7	-.4	.8*

Note For BL to MBW/BL with BL to MBW/BL df=256

For BL to MBW/BL with LAT to ELEV df = 6
* = P(<0.05)

blade width. The two angular measurements, the non-metric character (number of teeth) and the two ratios generally had weak ($r < 0.5$) but still significant relationships among themselves and with the other metric characters.

Both Kendall, and Spearman, rank correlations were calculated, but did not indicate any additional significant correlations among the variables. This provides good evidence for linear relationships among the variables within the morphological data set.

All of the morphological traits, except for the non-metric character, number of teeth, showed a negative relationship with latitude and longitude. This trend was reversed for elevation. The two significant univariate correlations between morphological traits and environmental variables were between leaf base angle and latitude and between the ratio MBW/BL and elevation.

In general, the northern and western populations had smaller leaves than those in the south and east. Leaf base angle (BA) becomes more accute from south to north (Table 5). Populations originating in the south and east have the point of maximum blade width closer to the center of the leaf than those in the north and west.

One notable exception to this general trend of decreased size with latitude and longitude occured in the Rocky Mountain House population (number 3). It had the longest and widest leaves, but it also had the largest coefficient of variation for these two traits (over twice as

large as the other populations), indicating that there was considerable variation in that sample.

Multivariate analyses

Using the populations sampled to define groups, a discriminant analysis was performed on the corrected, pooled within-groups variance-covariance matrix of the ten morphological traits. All pairwise group comparisons were significant ($p < 0.05$) when tested by an F ratio of the Mahalanhobis D^2 between groups (Green 1978). All ten morphological variables were included in the canonical discriminant functions.

A total of five significant ($p < 0.05$) canonical discriminant functions, which accounted for 99.35 percent of the total variation, were extracted from the data (Table 6).

The first morphological discriminant function, MDF1, accounted for 72 percent of the total variation. Of this 72 percent, 84 percent was explained by the groups' contribution. This first function formed a contrast between leaf blade width, point of maximum blade width and vein angle, which loaded positively, and leaf blade length and leaf blade width-length ratio, which loaded neagatively (Table 7). The first dimension seemed to separate the populations primarily along a north-south gradient and secondarily along an elevational gradient. The southern populations (numbers 1 and 2) scored high and positive on

Table 6. Summary of the discriminant analysis using the populations sampled as groups and the corrected variance-covariance matrix of ten morphological traits as input variables, for eight natural populations of trembling aspen from Alberta.

FUNCTION	EIGENVALUE	PER CENT OF VARIANCE	CUMULATIVE PER CENT	CANONICAL CORRELATION	CHI SQ.	PROB.
1	5.19	72.0	72.0	0.92	843	0.000
2	0.97	13.5	85.5	0.70	390	0.000
3	0.56	7.7	93.2	0.60	221	0.000
4	0.25	3.4	96.6	0.45	56	0.000

Table 7. Standardized canonical discriminant function coefficients for the corrected variance-covariance matrix of ten leaf morphology traits measured on eight natural trembling aspen populations sampled in Alberta as groups.

	DISCRIMINANT FUNCTION				
TRAIT	MDF1	MDF2	MDF3	MDF4	MDF5
BL	-6.4	2.1	4.4	1.4	3.1
BW	3.7	-0.1	-3.2	-2.1	-1.9
MBW	2.0	-1.7	0.6	0.5	-0.3
PL	0.7	-0.2	-0.7	-0.1	-0.9
BA	0.5	0.5	0.1	0.6	-0.0
VA	1.0	-0.1	0.0	-0.4	0.1
AL	0.6	-0.4	-0.7	-0.6	-0.2
NOTETH	-0.1	-0.6	0.2	0.4	-0.5
BWBL	-3.0	0.5	2.7	1.1	0.9
MBWBL	0.4	0.1	-0.5	-0.5	0.3

MDFi refers to the i *th* morphological dicriminant function

this axis, while mid-latitude and northern populations scored near zero or negative (Figure 5). Therefore, the southern populations tended to have wider, shorter leaves, with the point of maximum width nearer the the middle of the leaf and with large base vein angles as compared to the mid-latitude and northern populations. The most important morohological trait separating these populations was leaf blade length.

The second discriminant function accounted for 13.5 percent of the remaining variation; 50 percent of this variation came from the groups' contribution. This second function was primarily a contrast between leaf blade length and leaf base angle, which loaded positively, and point of maximum leaf blade width and number of teeth, which loaded

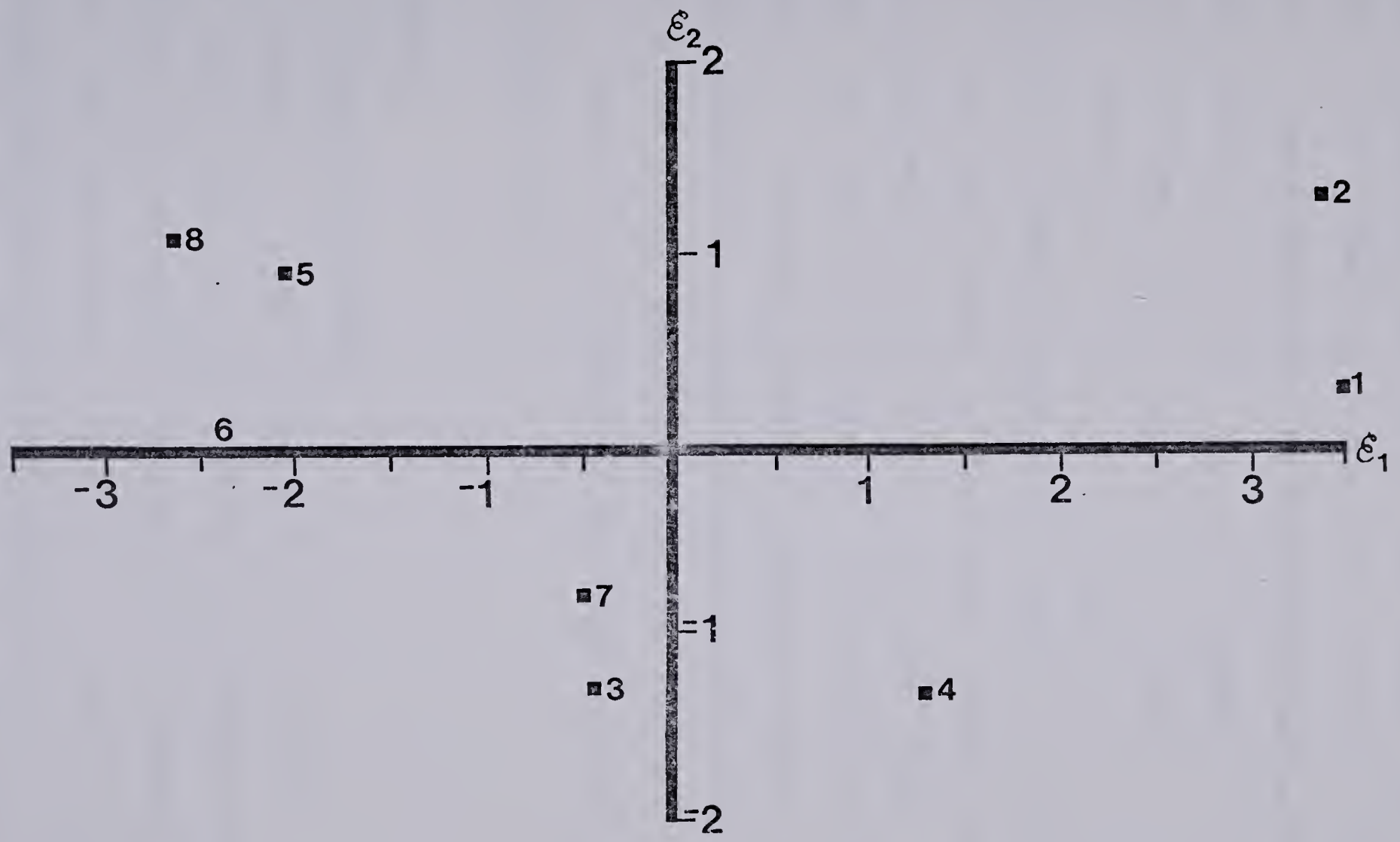

Figure 5. Population centroids (■) on the first two discriminant axes ($\mathcal{E}_1$ and $\mathcal{E}_2$) of morphology for eight natural trembling aspen populations in Alberta.

negatively. Thus, to score positive on this dimension, individuals within a population would have to score higher on leaf blade width and leaf base angle than point of maximum leaf blade width and number of teeth. There was no apparent simple geographic relationships indicated by this second dimension. Rather, this axis seemed to be separating within the three major population clusters (1,2; 3,4,7; 5,6,8) indicated in the first dimension. For example, the trees in the Lundbreck population (number 2) would have to score relatively higher on leaf blade length and leaf blade angle, and lower on point of maximum leaf blade width and number of teeth than trees in the Cypress Hills population (number 1). Therefore, trees at Lundbreck would tend to have longer leaves with a larger leaf base angle, fewer teeth per side of leaf blade, and the point of maximum leaf blade width closer to the base of the leaf relative to trees at the Cypress Hills.

B. Genetic

Univariate analyses

Seventy-six alleles coding for 26 putative structural gene loci were recorded in the seven populations studied (Table 8). Including the alleles with a frequency less than 5% in any population, a total of 103 putative alleles were found segregating in the seven populations.

With the exception of APH1 and PER5, variant alleles were found in every enzyme system studied, but not in every

Table 8. Allelic variation at 26 putative gene loci in seven natural populations of trembling aspen in Alberta.

			POPULATION						
ENZYME	LOCUS	ALLELE	1	2	3	4	5	6	7
AAT	1	A1	.00	.07	.00	.00	.00	.02	.04
		A2	.44	.77	.45	.78	.68	.55	.68
		A3	.56	.17	.55	.22	.32	.43	.29
E(h)			.49	.38	.49	.34	.43	.51	.46
O(h)			.44	.47	.43	.37	.63	.77	.60
AAT	2	A1	.94	.98	.97	.95	.90	.90	.94
		A2	.00	.02	.00	.02	.07	.10	.01
		A3	.06	.00	.03	.03	.03	.00	.05
E(h)			.12	.03	.06	.10	.18	.18	.12
O(h)			.00	.03	.00	.10	.20	.20	.12
ALP	1	A1	.61	.18	.50	.03	.10	.00	.00
		A2	.39	.82	.50	.97	.90	1.00	1.00
E(h)			.48	.30	.50	.06	.18	.00	.00
O(h)			.41	.37	1.00	.07	.13	.00	.00
APH	1	A1	1.00	1.00	1.00	1.00	1.00	1.00	1.00
E(h)			.00	.00	.00	.00	.00	.00	.00
O(h)			.00	.00	.00	.00	.00	.00	.00
APH	2	A1	.48	.85	.45	.55	.40	.40	.44
		A2	.52	.15	.55	.45	.55	.57	.55
		A3	.00	.00	.00	.00	.05	.03	.01
E(h)			.50	.26	.50	.50	.53	.52	.51
O(h)			.47	.18	.50	.77	.77	.47	.80
APH	3	A1	.00	.00	.00	.00	.05	.00	.00
		A2	.17	.60	.53	.42	.40	.45	.39
		A3	.83	.40	.47	.58	.45	.55	.61
		A4	.00	.00	.00	.00	.10	.00	.00
E(h)			.29	.48	.50	.49	.61	.50	.48
O(h)			.28	.47	.68	.30	.60	.43	.38
CAT	1	A1	.45	.50	.50	.48	.48	.52	.58
		A2	.55	.50	.50	.52	.52	.48	.42
E(h)			.50	.50	.50	.50	.50	.50	.49
O(h)			.47	.87	.53	.83	.77	.63	.55
CLE	2	A1	.00	.00	.00	.03	.05	.00	.00
		A2	.42	.57	.15	.45	.53	.42	.42
		A3	.58	.42	.85	.45	.33	.50	.58
		A4	.00	.02	.00	.07	.09	.08	.00
E(h)			.49	.50	.25	.59	.60	.57	.49
O(h)			.53	.43	.30	.50	.60	.64	.30

Table 8. *continued*

ENZYME	LOCUS	ALLELE	POPULATION 1	2	3	4	5	6	7
CLE	3	A1	.00	.00	.05	.00	.10	.00	.02
		A2	.38	.22	.30	.28	.47	.37	.19
		A3	.62	.78	.50	.62	.43	.60	.69
		A4	.00	.00	.15	.10	.00	.03	.10
E(h)			.47	.34	.64	.53	.58	.50	.48
O(h)			.50	.10	.53	.30	.33	.47	.40
CLE	4	A1	.00	.00	.05	.07	.00	.00	.00
		A2	.44	.38	.43	.47	.50	.50	.50
		A3	.56	.57	.37	.43	.50	.50	.50
		A4	.00	.05	.15	.03	.00	.00	.00
E(h)			.49	.53	.65	.59	.50	.50	.50
O(h)			.69	.67	.80	1.00	1.00	1.00	1.00
FLE	1	A1	.38	.45	.48	.45	.12	.48	.61
		A2	.62	.55	.52	.55	.88	.52	.39
E(h)			.47	.50	.50	.50	.21	.50	.48
O(h)			.69	.77	.70	.50	.23	.37	.72
FLE	2	A1	.50	.28	.40	.37	.15	.45	.42
		A2	.50	.72	.53	.63	.85	.55	.56
		A3	.00	.00	.07	.00	.00	.00	.02
E(h)			.50	.41	.55	.47	.25	.50	.50
O(h)			.31	.23	.47	.33	.23	.17	.58
FLE	4	A1	.50	.48	.40	.43	.43	.52	.45
		A2	.19	.45	.52	.50	.48	.40	.54
		A3	.31	.07	.08	.07	.08	.08	.01
E(h)			.62	.56	.57	.56	.57	.57	.51
O(h)			1.00	.97	.90	.97	.97	.97	.85
B-GLD	1	A1	.16	.10	.52	.42	.42	.05	.39
		A2	.45	.50	.48	.52	.50	.68	.60
		A3	.39	.40	.00	.06	.08	.28	.01
E(h)			.62	.58	.50	.56	.57	.47	.49
O(h)			1.00	.67	.63	.97	.93	.67	.75
HEA	1	A1	.47	.42	.53	.42	.45	.58	.54
		A2	.34	.57	.47	.58	.50	.40	.46
		A3	.19	.01	.00	.00	.05	.02	.00
E(h)			.63	.50	.50	.48	.54	.50	.50
O(h)			.97	.47	.47	.90	.70	.57	.58

Table 8. *continued*

ENZYME	LOCUS	ALLELE	POPULATION 1	2	3	4	5	6	7
LAP	1	A1	.45	.63	.52	.53	.47	.60	.48
		A2	.55	.37	.48	.47	.53	.40	.52
E(h)			.50	.46	.50	.50	.50	.48	.50
O(h)			.59	.73	.57	.73	.93	.47	.95
LAP	2	A1	.31	.47	.38	.68	.48	.00	.52
		A2	.69	.53	.62	.32	.52	1.00	.48
E(h)			.43	.50	.47	.43	.50	.00	.50
O(h)			.38	.47	.50	.57	.97	.00	.70
LAP	3	A1	.42	.38	.40	.47	.45	.50	.38
		A2	.58	.55	.60	.52	.50	.45	.62
		A3	.00	.07	.00	.01	.05	.05	.00
E(h)			.49	.55	.48	.51	.54	.54	.47
O(h)			.72	.90	.33	.97	1.00	.87	.65
PER	1	A1	.52	.43	.45	.47	.43	.48	.45
		A2	.48	.57	.55	.53	.57	.52	.55
E(h)			.50	.49	.50	.50	.49	.50	.50
O(h)			.59	.33	.50	.53	.47	.50	.65
PER	4	A1	.09	.05	.00	.00	.19	.10	.04
		A2	.50	.52	.45	.47	.48	.47	.42
		A3	.41	.43	.55	.53	.33	.43	.54
E(h)			.58	.54	.50	.50	.61	.58	.53
O(h)			1.00	.77	.77	.87	.93	1.00	.82
PER	5	A1	1.00	1.00	1.00	1.00	1.00	1.00	1.00
E(h)			.00	.00	.00	.00	.00	.00	.00
O(h)			.00	.00	.00	.00	.00	.00	.00
PGI	1	A1	.00	.15	.13	.12	.50	.42	.50
		A2	1.00	.85	.87	.88	.50	.58	.50
E(h)			.00	.26	.23	.21	.50	.47	.50
O(h)			.00	.30	.28	.23	1.00	.87	1.00
PGI	2	A1	.00	.00	.00	.00	.00	.00	.05
		A2	.53	.40	.47	.60	.50	.60	.80
		A3	.47	.60	.53	.40	.50	.40	.15
E(h)			.50	.48	.50	.48	.50	.48	.34
O(h)			.56	.53	.33	.40	.80	.67	.35

Table 8. *continued*

ENZYME	LOCUS	ALLELE	POPULATION 1	2	3	4	5	6	7
PGI	3	A1	.00	.00	.08	.00	.00	.00	.00
		A2	.50	.77	.53	.63	.72	.65	.56
		A3	.50	.23	.10	.37	.28	.35	.44
		A4	.00	.00	.29	.00	.00	.00	.00
E(h)			**.50**	**.36**	**.62**	**.46**	**.41**	**.46**	**.49**
O(h)			**.50**	**.33**	**.40**	**.37**	**.57**	**.70**	**.78**
PGM	1	A1	1.00	.82	.83	.97	.80	.65	.75
		A2	.00	.15	.10	.03	.13	.23	.04
		A3	.00	.03	.07	.00	.07	.08	.18
		A4	.00	.00	.00	.00	.00	.04	.03
E(h)			**.00**	**.31**	**.29**	**.06**	**.34**	**.52**	**.30**
O(h)			**.00**	**.37**	**.33**	**.10**	**.40**	**.27**	**.50**
SOD	1	A1	.45	.37	.52	.45	.55	.43	.51
		A2	.34	.57	.32	.43	.37	.45	.32
		A3	.21	.06	.16	.12	.08	.12	.17
E(h)			**.64**	**.54**	**.61**	**.60**	**.56**	**.60**	**.61**
O(h)			.56	.57	.77	1.00	.33	.83	.62

E(h) = expected heterozygosity
O(h) = observed heterozygosity

population. However, if an allele's frequency was not greater than or equal to 5 percent in at least one population, it was pooled with the allele closest in migration distance. This was done because of the constraints on accuracy from the sample size selected from Appendix 1.

Local fixation or loss of an allele within a population occurs in the following systems; ALP1A1 (populations 6 and 7) *B*-GLD1A3 (population 3), LAP2A2 (population 6) and PGI1A2 and PGM1A1 (population 1). Locally rare variants occured in the following systems: APH3A1 and A4 (population 5), PGI2A1

Table 9. Summary of the chi-square analysis of allele frequency homogeneity for twenty-four putative structural gene loci in seven natural trembling aspen populations in Alberta.

LOCUS	D.F.	X^2
AAT1	12	25.75*
AAT2	12	12.63
ALP1	6	75.54*
APH2	12	22.92*
APH3	18	40.25*
CAT1	6	1.27
CLE2	18	33.25*
CLE3	18	33.21*
CLE4	18	30.27*
FLE1	6	18.55*
FLE2	12	21.37*
FLE4	12	25.53*
B-GLD1	12	57.91*
HEA1	12	24.36*
LAP1	6	3.56
LAP2	6	34.65*
LAP3	12	8.63
PER1	6	0.68
PER4	12	14.84
PGI1	6	42.71*
PGI2	12	26.13*
PGI3	18	83.53*
PGM1	18	33.95*
SOD1	12	8.72

* indicates significant at $p < 0.05$

(population 7) PGI3A1 and A4 (population 3). In addition, there are numerous examples of two or more populations having the same rare variant, e.g., APH2A1 (populations 5,6,7), CLE4A4 (populations 2,3,4), PGM1A4 (populations 6 and 7). Several of the polymorphic loci had one allele predominant or exhibited little among-population differences in allele frequencies (e.g., AAT2, CAT1, CLE4, LAP3, PER1, PER4). The remaining 18 loci showed either broad regional variability (e.g., *B*-GLD, PGI) or strong intra-population

variability with no single allele predominating (e.g., APH3, CLE2). A chi-square analysis for homogeneity of allele frequency across the populations by gene locus indicated that only 6 loci (AAT2, CAT1, LAP1, LAP3, PER1 and SOD1) had homogenious gene frequencies across the seven populations (Table 9). The X^2 values ranged from 83.53 (PGI3) to 0.68 (PER1). This range in X^2 values for individual loci suggests that selection, drift or some other factor is acting differentially among the loci.

Approximately 43 percent of the loci tested showed a significant deviation from the expected Hardy-Weinberg equilibrium proportions (Table 10). Twenty-five percent of these departures were due to a heterozygote deficiency, with the remainder due to an excess of heterozygotes. Only 4 of the loci scored (CLE4, FLE4, *B*-GLD1 and PER4) showed a consistent direction (excess) of departure from expected Hardy-Weinberg proportions. Most of the departures within these systems were significant for each population.

Several facts are evident from the summary of the genetic data given in Table 11:

(i) trembling aspen is an extremely variable species, whether measured by expected or observed heterozygosity, average number of alleles per locus or the percentage of polymorphic loci; and

(ii) in all populations, the observed proportion of heterozygotes always exceeded the expected number of heterozygotes.

Table 10. Hardy-Weinberg expectations using a maximum likelihood G^2 statistic, with Levene's(1949) adjustment for small sample size, for the genetic data on seven natural trembling aspen populations in Alberta.

		POPULATION						
ENZYME	PAR**	1	2	3	4	5	6	7
AAT1	df	1	3	1	1	1	3	3
	G^2	0.5	4.0	0.6	0.1	8.7*	9.5*	6.4
	E(h)	-	+	-	+	+	+	+
AAT2	df	1	1	1	3	3	1	3
	G^2	16.1*	0.0	10.5*	0.1	0.6	0.6	7.2
	E(h)	-	=	-	+	+	+	+
ALP1	df	1	1	1	1	1	0	0
	G^2	0.8	2.3	40.5*	0.0	1.7	0.0	0.0
	E(h)	-	+	+	=	-	0	0
APH2	df	1	1	1	1	3	3	3
	G^2	0.2	3.2	0.0	9.2*	9.0*	4.1	16.3*
	E(h)	-	-	-	+	+	-	+
APH3	df	1	1	1	1	1	1	3
	G^2	0.0	0.0	3.2	0.0	3.6	0.6	8.6*
	E(h)	-	-	+	-	+	-	-
CAT1	df	1	1	1	1	1	1	1
	G^2	0.2	17.2*	0.0	8.9*	10.7*	1.9	0.5
	E(h)	-	+	+	+	+	+	+
CLE2	df	1	3	1	6	6	3	1
	G^2	0.2	1.9	1.4	5.2	3.3	2.2	6.5*
	E(h)	+	-	+	-	-	+	-
CLE3	df	1	1	6	3	3	3	6
	G^2	0.1	13.9*	5.4	19.6*	14.8*	5.8	11.7
	E(h)	+	-	-	-	-	-	-
CLE4	df	1	3	6	6	1	1	1
	G^2	4.9*	3.7	15.9*	41.2*	40.6*	40.6*	54.5*
	E(h)	+	+	+	+	+	+	+
FLE1	df	1	1	1	1	1	1	1
	G^2	7.5*	9.2*	4.6*	0.0	0.1	2.4	12.3*
	E(h)	+	+	+	-	+	-	+
FLE2	df	1	1	3	1	1	1	3
	G^2	5.0*	5.6*	1.7	2.7	0.3	15.0*	1.8
	E(h)	-	-	-	-	-	-	+

Table 10. *continued*

ENZYME	PAR**	POPULATION 1	2	3	4	5	6	7
FLE4	df	3	3	3	3	3	3	3
	G^2	4.3	33.8*	22.4*	29.1*	41.7*	33.2*	23.6*
	E(h)	+	+	+	+	+	+	+
B-GLD1	df	3	3	1	3	3	3	3
	G^2	32.7*	8.7*	1.9	33.2*	34.5*	10.0*	14.2*
	E(h)	+	+	+	+	+	+	+
HEA1	df	3	3	1	3	3	3	1
	G^2	24.9*	1.4	0.2	24.8*	3.1	1.5	0.8
	E(h)	+	-	-	+	+	+	+
LAP1	df	1	1	1	1	1	1	1
	G^2	1.1	13.0*	0.4	6.6*	28.5*	0.1	41.2*
	E(h)	+	+	+	+	+	-	+
LAP2	df	1	1	1	1	1	0	1
	G^2	0.7	0.2	0.1	2.9	33.2*	0	6.3*
	E(h)	-	-	+	+	+	0	+
LAP3	df	1	3	1	3	3	3	1
	G^2	7.2*	24.8*	3.1	33.2*	40.6*	18.2*	6.1*
	E(h)	+	+	-	+	+	+	+
PER1	df	1	1	1	1	1	1	1
	G^2	1.0	3.5	0.0	0.1	0.1	0.0	3.7
	E(h)	+	-	-	+	-	-	+
PER4	df	3	3	1	1	3	3	3
	G^2	43.3*	10.0*	9.2*	18.1*	22.1*	33.5*	18.1*
	E(h)	+	+	+	+	+	+	+
PGI1	df	0	1	1	1	1	1	1
	G^2	0	1.4	1.1	0.8	40.6*	21.7*	54.4*
	E(h)	0	+	+	+	+	+	+
PGI2	df	1	1	1	1	1	1	3
	G^2	0.4	0.3	3.7	1.0	11.0*	4.4*	1.6
	E(h)	+	+	-	-	+	+	+

Table 10. *continued*

ENZYME	PAR**	POPULATION 1	2	3	4	5	6	7
PGI3	df	1	1	6	1	1	1	1
	G^2	0.0	0.2	20.0*	2.1	6.5*	9.9*	13.9*
	E(h)	+	-	-	-	+	+	+
PGM1	df	0	3	3	1	3	6	6
	G^2	0	2.3	1.8	0.1	2.8	17.4*	6.5*
	E(h)	0	+	+	+	+	-	+
SOD1	df	3	3	3	3	3	3	3
	G^2	6.0	0.6	16.0*	31.9*	6.4	9.6*	12.2*
	E(h)	-	+	+	+	-	+	+

* indicates significant P(<0.05) deviation
PAR**
df = degrees of freedom
G^2 = maximum liklihood estimate (assomtotic X^2)
E(h): + = excess of heterozygotes;
- = deficiency of heterozygotes.

The analysis of gene diversity shown in Table 12 indicates that, although the total gene diversity is quite high, most of this diversity resides within populations. The values of inter-population differentiation G(st) vary from 0 to 0.331 over the 26 loci investigated. The mean over all the loci for G(st) shows that about 6 percent of the detected genic variation is due to inter-population differentiation. Several loci (ALP1, LAP2 and PGI1) did show large among-population differentiation.

Virtually the same results obtain when genetic distance between populations is considered (Table 13). The genetic distances ranged from 0.026 to 0.089 with an overall mean of

Table 11. Summary of the descriptive population statistics from the genetic data measured on seven natural populations of trembling aspen in Alberta.

POPULATION	N	E(h)	O(h)	Av. # alleles per locus	%loci polymorphic
1	32	.41	.49	2.0	85
2	30	.40	.46	2.3	88
3	30	.44	.49	2.3	88
4	30	.41	.53	2.3	84
5	30	.43	.60	2.5	92
6	30	.42	.52	2.3	85
7	40	.42	.56	2.4	88

where N = sample size and E(h) and O(h) are the observed and expected heterozygosities respectively.

0.059. The Cypress Hills population (number 1) was most different from all other populations, while the Nordegg population (number 4) was most similar to all other populations. In addition, there was a significant ($p < 0.05$) positive correlation between the genetic distance and geographic distance between populations.

A phylogenetic tree of the seven aspen populations sampled shows a major subdivision between populations 1 and 3 (Cypress Hills and Rocky Mountain House) and the remaining five poplations sampled (Figure 6). Because of the limited number of populations sampled, it is probably wise not to extrapolate beyond the first major subdivision.

Multivariate analyses

A discriminant analysis, using the populations sampled to define groups was perfomed on the individuals, using the most common allele of every variable gene locus. Across the

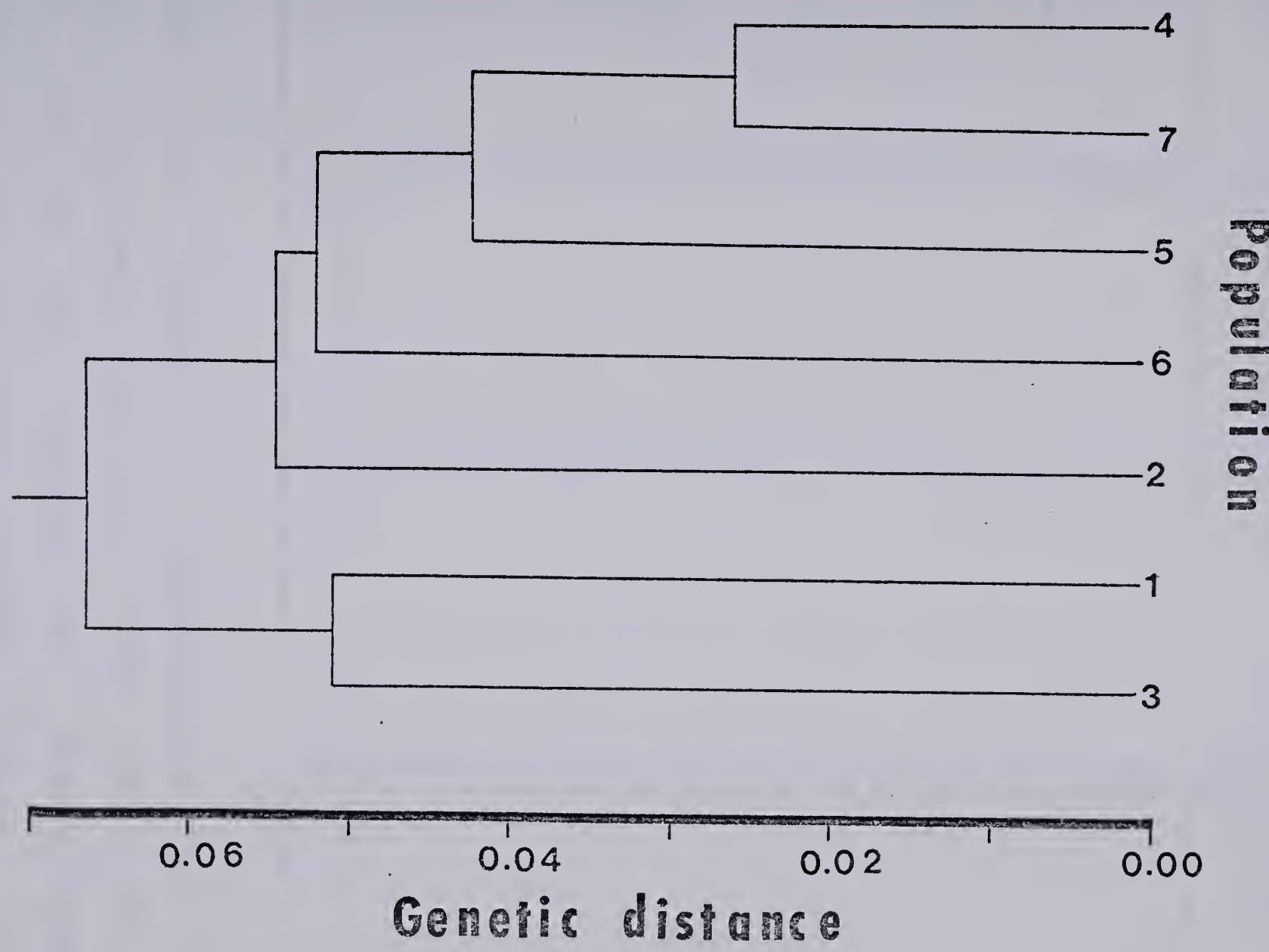

Figure 6. Phylogenetic tree of seven natural trembling aspen populations from Alberta. The dendrogram was constructed according to the unweighted pair-group method of Sneath and Sokal (1973), using the genetic distances presented in Table 13.

Table 12. Analysis of gene diversity and proportion of population subdivision at 26 gene loci among seven natural populations of trembling aspen in Alberta.

GENE LOCUS	TOTAL GENE DIVERSITY (Ht)	GENE DIVERSITY WITHIN POPULATIONS (Hs)	PROPORTION OF INTERPOPULATION DIFFERENTIATION (Gst)
AAT1	.48	.44	.08
AAT2	.12	.11	.02
ALP1	.32	.22	.33
APH1	.00	.00	.00
APH2	.51	.47	.08
APH3	.51	.48	.07
CAT1	.50	.49	.01
FLE1	.49	.45	.08
FLE2	.48	.45	.05
FLE4	.59	.56	.04
CLE2	.54	.50	.08
CLE3	.53	.51	.04
CLE4	.55	.54	.02
B-GLD1	.60	.54	.10
HEA1	.54	.52	.02
LAP1	.50	.49	.02
LAP2	.48	.40	.16
LAP3	.52	.51	.01
PER1	.50	.50	.00
PER4	.56	.55	.02
PER5	.00	.00	.00
PGI1	.38	.31	.19
PGI2	.50	.47	.06
PGI3	.51	.47	.07
PGM1	.30	.28	.07
SOD1	.60	.59	.02
MEAN	.45	.42	.06

groups, the following loci (represented by only 1 allele) had non-significant univariate F ratios: AAT2, CAT1, HEA1, PER1, PER4 and SOD1. However, only three of these loci (AAT2, CAT1 and PER1) were excluded from the canonical discriminant function. All 21 pairwise group comparisons were significant ($p < 0.05$) when tested by an F ratio of the Mahalanobis D^2 between groups (Green 1978). Groups

covariance matricies were significantly different from each other ($p < 0.05$) when tested with Box's M (Cooley and Lohenes 1962). This heterogeniety of group covariance matricies is analogous to the heterogeniety of variances among treatments in the univariate ANOVA.

A total of five significant ($p < 0.05$) canonical discriminant functions were derived from the input data. (Table 14). Using these five functions, 80 percent of the individuals could be correctly re-classified into their original populations.

The first canonical discriminant function, GDF1, accounted for 55 percent of the total variation in the data set. Seventy-seven percent of this 55 percent came from the groups' contribution. This first function could be labelled PGI1 and ALP1. The internal structure of this dimension consisted primarily of a contrast between ALP1A2 and CLE3A3, which loaded positively and PGI1A2 and PGM1A1 which loaded negatively (Table 15). Therefore, to score positive on this axis, individuals within a population would have to score high on ALP1A2 and CLE3A3 and low on PGI1A2 and PGM1A1. Thus, the northern populations (numbers 5, 6 and 7) tended to have higher frequencies of ALP1A2 and CLE3A3 and lower frequencies of PGI1A2 and PGM1A1, relative to the central and southern populations in this study. The most important loci discriminating among these populations were PGI1 and ALP1.

The second canonical discriminant function accounted

Table 13. Genetic distance (Nei 1972) (below the principal diagonal) and geographic distance (Km) (above the principal diagonal) for pairwise comparisons between the seven natural trembling aspen populations from Alberta.

	POPULATION						
	1	2	3	4	5	6	7
1	-	300	460	540	700	740	520
2	.074	-	330	360	510	670	450
3	.051	.071	-	100	260	360	170
4	.067	.031	.051	-	180	380	230
5	.087	.054	.071	.036	-	330	310
6	.072	.056	.070	.060	.051	-	260
7	.089	.064	.062	.026	.047	.042	-

The correlation between genetic distance and geographic distance = 0.49*.

Table 14. Summary of the discriminant analysis using the populations sampled as groups with the common allele at every polymorphic gene locus as input variables, for seven natural populations of trembling aspen from Alberta.

FUNCTION	EIGENVALUE	PER CENT OF VARIANCE	CUMULATIVE PER CENT	CANONICAL CORRELATION	CHI SQ.	PROB.
1	3.38	55.4	55.4	0.87	430	0.000
2	1.01	16.7	72.1	0.71	286	0.000
3	0.70	11.5	83.6	0.64	175	0.000
4	0.54	8.8	92.4	0.59	86	0.000
5	0.28	4.7	92.1	0.47	15	0.005

Table 15. Standardized canonical discriminant function coefficients for the twenty-four polymorphic gene loci (using one allele per locus) defining the seven natural trembling aspen populations sampled as groups.

	DISCRIMINANT FUNCTION				
ALLELE	GDF1	GDF2	GDF3	GDF4	GDF5
AAT1A3	-0.2	0.4	0.0	-0.1	-0.1
ALP1A2	0.7	-0.3	-0.1	0.2	-0.3
APH2A1	0.0	-0.6	-0.2	-0.2	0.2
APH3A3	-0.0	0.1	0.2	0.3	-0.5
CLE2A2	0.1	-0.4	-0.1	-0.0	-0.3
CLE3A3	0.2	-0.2	-0.1	0.1	0.0
CLE4A2	0.1	0.1	0.2	0.0	-0.1
FLE1A2	-0.0	0.2	0.2	-0.6	-0.5
FLE2A1	-0.1	0.1	-0.1	0.1	-0.2
FLE4A1	0.0	-0.1	-0.3	-0.0	-0.2
B-GLD1A2	0.1	-0.1	-0.2	0.3	-0.1
HEA1A1	-0.0	0.2	-0.0	0.2	0.1
LAP1A1	-0.0	-0.1	-0.2	0.0	0.1
LAP2A2	-0.2	0.3	-0.8	-0.1	-0.2
LAP3A2	0.1	0.0	0.1	0.2	0.2
PER4A2	-0.1	-0.2	-0.1	-0.2	-0.1
PGI1A2	-0.7	-0.3	-0.2	0.3	-0.2
PGI2A2	-0.0	0.2	0.3	0.4	-0.1
PGI3A2	0.0	-0.1	-0.1	-0.4	-0.0
PGM1A1	-0.2	-0.2	0.3	0.1	-0.4
SOD1A1	0.2	0.1	0.1	-0.0	-0.0

where GDFi refers to the i *th* genetic discriminant function

for 17 percent of the remaining variation in the data set. Fifty percent of this 17 was from the groups' contribtuion. This second function could be labeled APH2. This dimension consisted of a contrast between AAT1A3, LAP2A2 and FLE1A2, which loaded positively, and APH2A1, CLE2A2, PGI1A2 and ALP1A2, which loaded negatively. Thus, the Rocky Mountain and foothills populations (numbers 2 and 4) would tend to have higher frequencies of APH2A1, CLE2A2, PGI1A2 and ALP1A2 and lower frequencies of AAT1A3, LAP2A2 and FLE1A2 relative

to the grassland populations (numbers 1 and 3) and the Boreal populations (numbers 5, 6 and 7; Figure 7).

Relationships between genetics and morphology

Relationships between the morphological and genetic data sets were examined by canonical correlation. Input data consisted of all ten morphological traits and the common allele of every polymorphic gene locus. Three significant ($p < 0.05$) canonical correlation functions were extracted from these data. All of the morphological variables were included, but the following gene loci were excluded because they did not load > |.2| on any of the genetic canonical equations: AAT1, AAT2, CAT1, CLE4, FLE4, *B*-GLD1, HEA1, LAP1 and SOD1.

The sets of canonical equations for the three significant canonical functions are shown in equations 1a - 3b. Included is the canonical correlation (i.e., the square root of the eigenvalue associated with that dimension) between the morphological and genetic data sets within a function.

SET 1 ($\sqrt{\lambda_1} = 0.68$)

$$G1 = ALP1A2 - (PG11A2 + PGM1A1 + PGM1A1 + APH2A1 + PER4A2 + CLE2A1) \quad 1a$$

$$M1 = BL + NOTETH + BWBL\ (MBW + BA + VA) \quad 1b$$

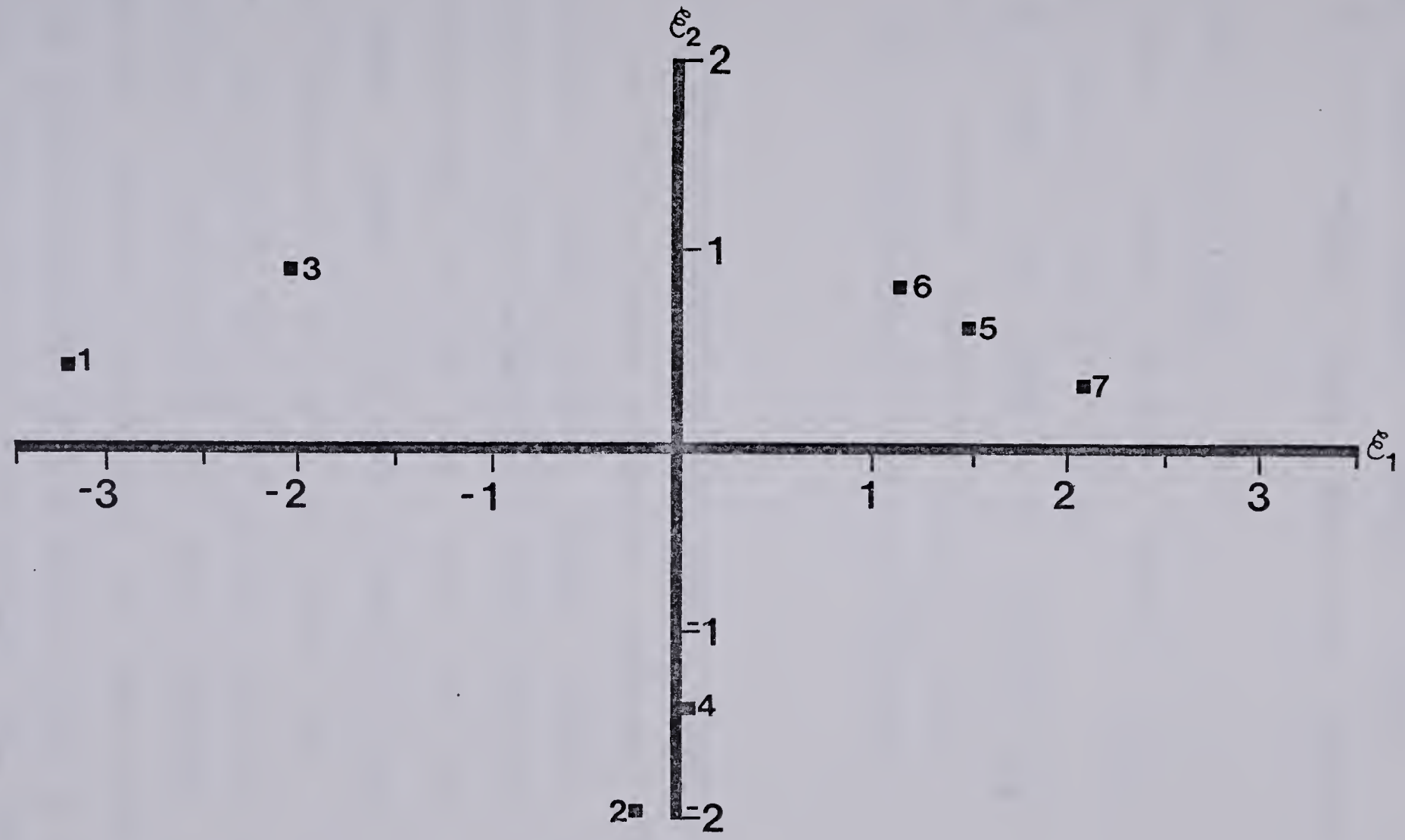

Figure 7. Population centroids (■) on the first two discriminant axes ($\hat{\xi}_1$ and $\hat{\xi}_2$) of the genetic data for seven natural trembling aspen populations in Alberta.

SET 2 ($\sqrt{\lambda_2} = 0.57$)

G2 = ALP1A2 + FLE1A2 + PGM1A1 + FLE2A1 + PGI1A2 + LAP2A2 + PGI3A2 + APH2A1 + APH3A3 - (LAP3A2 + CLE3A3) 2a

M2 = BW + AL + MBWBL - (MBW + BWBL + BL + NOTETH) 2b

SET 3 ($\sqrt{\lambda_3} = 0.53$)

G3 = CLE2A2 + LAP2A2 + APH2A1 - (PER1A1 + PGI2A2 + PGM1A1 + APH3A3 + CLE3A3 + ALP1A2 + PGI1A2) 3a

M3 = BL + BWBL - (AL + MBWBL + PL + NOTETH + BW) 3b

where: Gi = genetic canonical equation i and

Mi = morphological canonical equation i

The correlation between the two linear transformations in set 1 is 0.68. These equations indicated that, as the frequencies of PGI1A2, CLE2A1, PGM1A1, APH2A1 and PER4A2 increased and the frequency of ALP1A2 decreased, leaf blade length and number of teeth per side of leaf blade increased. The leaves tended to be more rounded with more acute base and vein angles with the point of maximum leaf blade width nearer the base of the leaf. The most important genetic and morphological relationship in this dimension was that between PGI1 and point of maximum leaf blade width.

The second dimension of this analysis had a significant canonical correlation of 0.57 between the genetic and morphological data. The most important relationship indicated by these two equations was between ALP1 and leaf

blade width. These equations showed that individuals with higher scores of ALP1A2, FLE1A2, PGM1A1, FLE2A1, PGI1A2, LAP2A2, PGI3A2, APH2A1 and APH3A3 and lower scores of LAP3A2 and CLE3A2 tended to have wider, shorter leaves with longer apicies and fewer teeth per side of leaf blade. Additionally, the leaves tended to be more elipsoid and less obovate in shape than those individuals with recprical scores on these same alleles.

The third and final set of significant canonical functions derived for the genetic-morphological data had a canonical correlation of 0.53. These equations showed that, as the frequencies of CLE2A2, LAP2A2 and APH2A1 increased and the frequencies of PER1A1, PGI2A2, PGI1A1 APH2A3, CLE3A3, ALP1A2 and PGI1A2 decreased, these individuals tended to have long, narrow leaves, which were more obovate in shape with shorter leaf apices and petioles and fewer teeth per side of leaf blade than those individuals with recprical frequencies of these alleles.

IV. DISCUSSION

A. Morphology

Woody plant species of the north temperate zone are known to exhibit genetically-based differences in growth, phenology and morphology along north - south gradients, apparently related to the photoperiodic regime at each point along the gradient (Vaartaja 1959, 1960). Therefore, it is not surprising to find changes in leaf morphology along north-south gradients, as indicated by the correlation and discriminant analyses. Barnes (1975), in a large study of phenotypic variation in western aspen (from Utah, Montana, Oregon, Colorado, Washingtion, British Columbia and Idaho), reported results similar to those obtained in this study. Specifically, he found that leaf size (blade width/blade length ratio) decreased and the number of teeth per side of leaf blade increased along a south - north gradient, east of the Continental Divide.

Descriptions of morphology taken from the discriminant analysis showed that several clones from the southern collections (Cypress Hills and Lundbreck) resembled those from Barnes' central Utah and Colorado collections. One collection site in Barnes' study (Glacier - East, Montana) is approximately 80 km from my Lundbreck collection. In overall morphology, however, the Lundbreck population was more similar to the Colorado and southern Utah collections than to the eastern Montana populations.

Barnes (1975) stated that a few of these extremely large aspen clones from Utah and Colorado appear to be essentially unchanged morphologically since Pliocene or Miocene times. These comparisons were made from descriptions of the extinct *Populus pliotremuloides* Axel. given by Axelrod (1941), Chaney (1938) and Wolfe (1964). The similarities in morphology shown by these southern clones in the present study and the observation that several of these clones were larger in size than those in more northern populations leads to the speculation that a few of them may be be considerably older (i.e., fewer sexual generations in recent geological time) than the large majority of aspen in Alberta.

The discontinuity shown in Figure 5 between the southern (numbers 1 and 2) and central populations (numbers 3,4,5,6,7 and 8) along the first dimension of the discriminant analysis probably reflects the discontinuous nature of the sampling procedure more than any other factor. However, it indicates to a certain extent the essentially continuous nature of the morphological variation observed. The clustering effect (1,2; 3,4,7 and 5,6,8) along similar latitudinal gradients suggested that at least two factors may be affecting these clusters. First, each cluster of populations may have been derived from a similar ancestral background. In this case, we can postulate that similar selective pressures and a lack of migration among populations are giving rise to these population clusters.

This will be discussed later with the genetic distance calculations. Secondly, these population clusters may be expressing genotype x environment interactions, as indicated by their similar latitudinal orientations. In this case, the environment would be the primary factor influencing morphology. Since the discriminant analysis of the isoenzyme data should be essentially free of environmental effects, a comparison of the two results should indicate if there is genotype x environment interaction. This will be discussed later in the discriminant analysis of the genic data.

B. Genic variation

The results of the X^2 analysis provide a significant test for the homogeneity of allelic frequencies among the populations. However, the results of this analysis do not differentiate among the various factors which could have caused this heterogeneity in allelic frequencies. This heterogeneity or homogeneity could result from selection, random genetic drift, differential migration among populations or perhaps the mode of natural reproduction. Inter-locus heterogeneity in the degree of deviation from expected values could result from various factors opposing or complimenting each other. For example, consider the effect of random genetic drift on a population with and without migration from outside populations. The results of

the chi-square analysis in this study showed that 18 of the loci studied (75%) showed significant ($p < 0.05$) deviation from the expected distribution. The inter-locus variability in X^2 values suggested that factors affecting this deviation are acting on the loci independently and possibly in different directions and magnitudes.

Regardless of how genic variation was measured, all measures indicated that trembling aspen was an extremely variable species. Hamrick *et al.* (1979) have synthesized a summary of genic variation as a function of plant life history characteristics. If the life history characteristics of trembling aspen (e.g., taxonomic status, geographic range, generation length, mode of reproduction etc.) are considered, aspen possesses many of the characteristics (e.g., long generation woody plant, both sexual and asexual reproduction, wind pollinated, extensive geographic range, etc.) associated with species exhibiting moderate to high levels of genic variation (e.g. average heterozygosity of 0.3 - 0.4).

Another mechanism which can explain these high observed levels of genic variation is the mode of natural reproduction. When reproduction is limited to strictly vegetative or parthenogenic means with absolutely no recombination, eventually each individual would be heterozygous at all gene loci (Nei 1975). This result obtains since the ultimate source of variation is mutation, not recombination between individuals.

However, trembling aspen does produce large quantities of viable seed annually. Furthermore, this seed is produced sexually, not apomictically, (Fechner 1972,1976, Winton 1968b) allowing recombination between individuals. In order to examine the effects of partial vegetative reproduction on the amount of variation maintained in a population, a model for a population with partial vegetative and sexual reproduction was developed.

The model was derived for a population of size N, assuming neutral alleles. The model was developed for both an infinite and finite number of alleles at a locus. One case will be considered in both of these situations.

Theory

Let;

φ = Prob(a(i1)=a(j1)) $i \neq j$

ψ = Prob(a(i1)=a(i2))

V = proportion of offspring produced vegetatively

u = mutation rate to new alleles

where a(ij) is the i *th* locus in an individual in generation n, having come from the j *th* individual in generation n-1

The general recurssion relationships for this model are;

$$\psi' = (1 - u)^2 (V\psi + (1 - V)\{1/N (1/2 + 1/2\psi) + (1 - 1/N)\varphi\})$$

$$\varphi' = (1 - u)^2 \, (1/N \, (1/2 + 1/2 \, \psi) + (1 - 1/N) \, \varphi) \qquad -5-$$

Situation 1. Finite alleles model

In the following section, we will drop the $(1 - u)^2$ terms from equations 4 and 5. This will now limit the number of alleles in the population by not allowing any mutation. The amount of variation maintained in a population at equilibrium can be examined by defining a Jacobian matrix and finding the eigenvalues of this matrix. This will indicate the rate of approach to homozygosity and the stability of the system at the equilibrium conditions.

Define the Jacobian (J) as the following matrix;

$$J = \begin{pmatrix} \frac{\partial \psi'}{\partial \psi} & \frac{\partial \psi'}{\partial \varphi} \\ \frac{\partial \varphi'}{\partial \psi} & \frac{\partial \varphi'}{\partial \varphi} \end{pmatrix}$$

Consider the case where $(1 - V) > 1/N$

Noticing that the terms that are 1/N or larger can be dropped from the $\partial \psi'$ equations since both λ's are approximately 1, we obtain the following Jacobian matrix

from equations 4 and 5.

$$J = \begin{pmatrix} V & (1 - V) \\ 1/2N & (1 - 1/N) \end{pmatrix} \qquad -6-$$

The characteristic equation of 6 is;

$$\lambda^2 - (V + 1 - 1/N)\lambda + \{V\ (1 - 1/N) - 1/2N\ (1 - V)\} = 0 \qquad -7-$$

To approximate a solution to this equation, substitute $\lambda = 1 - x$. Ignoring terms of $> O\ (1/N)$, we obtain the following equation;

$$-(1 - V)x + \{(1 - V)/2N\} = 0$$

The roots of this equation are;

$$x(1) = 1/2N,$$
$$x(2) = (1 + V)$$

therefore,

$$\lambda_1 = 1 - 1/2N \qquad -8a-$$
$$\lambda_2 = V \qquad -8b-$$

where λ_1 is the largest eigenvalue. For the system to be stable at equilibrium, has to be less than one in absolute value. This is obviously true, so the system is stable at equilibrium.

Equation 8a is the same as the result for random mating finite sexual populations (e.g., Crow and Kimura 1970). Therefore, if the rate of establishment by sexual means is greater than 1/N, the population behaves as a random mating sexual population.

Situation 2. Infinate alleles model

Consider (1 - V) O(1/N)

From equations 4 and 5 and the above consideration, it is clear that the answer we expect will be approximately 1/N. Therefore, we can neglect terms such as $1/N^2$, u/N, u^2, etc. Considering the equilibrium situation, expanding equations 4 and 5 with a little re-arrangement, we obtain the following equations;

$$\psi = \{(1 - V)/((1 - V) + 2u)\}\varphi \qquad -9-$$

$$\varphi = 1/(2 + 4Nu - \{(1 - V)/((1 - V) + 2u)\}\varphi \qquad -10-$$

From equation 9 ψ can be regarded as the expected population heterozygosity (E(h)) and φ as the observed heterozygosity (O(h)). The constant $(1 - V)/((1 - V) + 2u)$ is describing the effect of vegetative reproduction on

individual heterozygosity. The effective population size (Ne) is then given by equating $1/(1 + 4Neu)$ = eq. 10 and solving for Ne.

Several problems arise when trying to apply the survey results to the predictions from the model. Since the sampling distributions of Ψ and φ are unknown, a significance test can not be formulated to test homogeneity of the expected and observed heterozygosities. Furthermore, the model does not predict situations where the expected heterozygosity is less than the observed heterozygosity. Therefore, we can consider only those situations where φ is consistently greater than Ψ. Similarily a ratio of expected to observed heterozygosity has to be strictly < 1.

If we assume a mutation rate, an estimate of (1 - V) can be obtained for the loci which show a consistent excess of observed heterozygotes by re-arranging equation 9 in terms of Ψ / φ. In these estimates, the mutation rate per generation per locus will be assumed to be a constant, 4.5×10^{-6} (Tobari and Kojima 1972). The assumption is not valid (as discussed later), but should serve as a base from which estimates of the sexual establishment rate (1 - V) can be obtained. Based on these calculations, the amount of sexual reproduction ranges from 9.45×10^{-5} (PGI1, population 4) to 9.0×10^{-6} (CLE4, populations 5, 6 and 7 and PGI1, populations 5 and 7; Table 16). The mean estimates showed sexual reproduction decreased in the following series of populations: Lundbreck (population 2), Rocky Mountain House

Table 16. Estimates** of the proportion of seven trembling aspen populations established by sexual means.

	POPULATION						
GENE LOCUS	1	2	3	4	5	6	7
CLE4	2.21*	3.41	3.90	1.30	0.90	0.90	0.90
B-GLD1	1.47	5.80	3.46	1.23	1.43	2.12	1.70
FLE4	1.47	1.23	1.55	1.23	1.28	1.28	1.35
PER4	1.24	2.11	1.67	1.22	1.72	1.24	1.64
PGI1	-	5.85	4.14	9.45	0.90	1.06	0.90
Mean	1.60	3.68	2.94	2.89	1.25	1.32	1.30

*All values shown are $x10^5$.
**Estimates are based on a constant mutation rate ($4.5x10^{-6}$) and are caluclated from equation 9. The loci used to estimate the sexual establishment rate are those which showed a consistent excess of observed heterozygotes.

(population 3), Nordegg (population 4), Cypress Hills (population 1), Slave Lake (population 6), Devon (population 7) and Hinton (population 5).

These estimates need to be interpreted with great uncertainty since we have no estimates of the mutation rate for these loci in trembling aspen. I am unable to account for the lower frequency of sexual reproduction in populations 1, 5, 6 and 7. Perhaps the Lundbreck, Rocky Mountain House and Nordegg trembling aspen populations recently experienced increased success in sexual establishment, due to favourable weather conditions at the time of establishment.

The results of the model and survey data suggest that vegetative reproduction may have an effect on the amount of variation maintained in the trembling aspen populations

studied. The effect of vegetative reproduction would be to increase the variability observed in these populations. However, at this time we can not determine the magnitude of that effect.

The application of the results from the model to the experimental data are contingent upon the neo-classical or neutralist hypothesis of population structure. Within populations, the average expected heterozygosity was always less than the observed population heterozygosity. However, it is not clear that pooling the results for all loci is correct, since, the rate of mutation to neutral alleles varies with classes of enzymes (Gillespie and Kojima 1968). For example, enzymes using variable substrates, or substrates from the external environment, are consistently more variable than enzymes using specific substrates, or those using substrates produced within the cell (Johnson 1973). Only 4 loci (16 %) showed a consistent direction of deviation from Hardy-Weinberg equilibrium. All of these were heterozygote excesses and most deviations were significant within all populations for these loci. When heterozygote excesses are observed, selection for heterozygote superiority (e.g., Fincham 1972, Futuyama 1979, Johnson 1978 and Koehn 1969) is commonly invoked as an explanation. Strobeck (1979), however, has demonstrated that a neutral locus can appear to be heterotic (i.e., an excess of heterozygotes over the predictions from one locus theory) if it is in a genome within which only one locus is being

selected. This model was developed for a partial selfing population but might be applicable to the present study as a first approximation.

Another way in which heterozygote excess or deficiency can arise is by randomly generated linkage disequilibrium (Kimura and Ohta 1971, Lewontin 1974, Lewontin and Kojima 1960) between coupling (excess) or repulsed (deficiency) gametes.

If in fact vegetative reproduction has an effect on the amount of variability in these populations linkage disequilibrium could be an extremely important parameter to consider. This results from the fact that in an asexually reproducing population, all genes are completely linked (Nei 1975) and therefore are in the same equilibrium or disequilibrium at the time of establishment (Eshel and Feldman 1970, Felsenstein 1974, Maynard-Smith 1978) and, increased by mutation (Hill 1975). Therefore, on the basis of Hardy-Weinberg expectations, it is not clear whether the model presented can be accepted as being reasonably correct or incorrect. This results partially from the study itself (unknown physiological functions of the isoenzymes), and partially from the inadequacy or insufficient dimensionality of the present theories (Lewontin 1974).

To invoke selection on the isoenzymatic level, the metabolic functions of the enzyme need to be understood. For example, alcohol dehydrogenase is known to function in the regeneration of NADP from NADH, thereby facilitating further

glycolysis and maintaing a high level of free energy (i.e., ATP) production under anaerobic conditions (Lehninger 1975). Thus many studies have found either altered catalytic activities, changes in fitness or changes in allele frequency (e.g., Bijlsma-Meeles and van Delden 1974, Brown *et al.* 1974, Brown *et al.* 1976, Crawford 1969, Day *et al.* 1974, Efron *et al.* 1973, Francis *et al.* 1974, Gibson 1970, Kojima and Tobari 1969, Marshall *et al.* 1973, Morgan 1975, Schwartz 1969, 1971 and Vigue and Johnson 1973) directly associated with anaerobic conditions in both plants and animals. However, studies which find relationships between the frequency of an allele within an isoenzyme and some environmental parameter (e.g., Bergmann 1975, 1978, Johnson *et al.* 1969, Kahler *et al.* 1980, Kojima *et al.* 1972, Matsumura and Sakai 1972, Mitton and Koehn 1975, Nevo and Bar 1976, Nevo *et al.* 1979, Perkins 1972, Rockwood-Sluss *et al.* 1973 and Tomaszewski *et al.* 1973) have only the strength of the correlation to rely upon as their sole demonstration of selection. In these types of studies, it must be kept in mind that a statistically significant correlation does not necessarily imply a cause-effect relationship. For example, Karlin and Richer-Dyn (1976) have demonstrated non-random patterns of variation can result from stochastic events (i.e., random drift). Therefore, even if significant relationships were found between allele frequency and some environmental parameter, it would not necessarily render the present model incorrect.

When non-random mating patterns exist in a population (e.g., selfing), measures of population differentiation show that most of the genic-based variation is contained in different populations (Brown 1979). This result obtains from a minimum of three directions:

(i) restriction of migration among populations;

(ii) random drift within individual populations; and

(iii) occurence of new mutations which are essentially unique.

Genic-based differences could accumulate rapidly, both within and among asexual populations (Haigh 1978). This occurs through the operation of "Muller's Ratchet" (Muller 1964). Basically the mechanism states that, the progeny in a lineage can have no fewer mutations than the individual giving rise to them. The ratchet operates because there is no recombination among individuals within a population. Operation of the ratchet is most dramatic in small populations (Haigh 1978). This should result in large among-population differences reflected in the organization of the genic variation. However, individuals (i.e., clones) within a population are also accumulating unique mutational differences independently and at the same average rate as the population. Since there is no migration among individuals (i.e., recombination) within a population, the individuals with a population should be as different from each other as different populations. Additionally, evolution (i.e., gene frequency change) is retarded by a factor of

1/NUg, (where, N is the population size, U is the mutation rate and g is the number of generations), as compared to sexual populations(Crow and Kimura 1965). Therefore, despite the primarily asexual mode of reproduction, these populations should appear similar in their organization of inter and intra-population genetic variability to sexual populations with intra-population gene flow. In fact, these results are similar to those obtained from random-mating woody-plant sexual species (Yeh and Layton 1979). However, the high levels of variation are more characteristic of parthenogenic or asexual populations (Asher and Nace 1971, Atchley 1977, Hebert 1974, Mitter *et al.* 1979, Parker and Selander 1976, Parker *et al.* 1977, Saura *et al.* 1977, Turner *et al.* 1980, Vrijenhoek *et al.* 1977, 1978).

Patterns of genic variation

Discriminant analysis of genic data showed that PGI1 and ALP1 best distinguished among populations. Not suprisingly then, ALP1 and PGI1 also showed the greatest proportion of inter-population differentiation (Table 12).

The discriminant analysis of genetic data showed a weaker latitudinal arrangement of populations, than that obtained by the morphological results. The clinal pattern and clustering along latitudinal gradients indicated by the discriminant analysis of morphology suggests that when compared to these results for the genetic data, there are

possibly genotype x environment interactions. This conclusion was reached because the genetic data, which showed different clustering, should be free of environmental effects. This supports the earlier suggestion of genotype x environment interaction based on the morphological results.

Despite the fact that there was only about 6 per cent genic differentiation among populations, 18 percent more individuals were correctly classified by discriminant analysis of the isoenzyme data than were classified by the same analysis of morphological data.

Studies on the Yanomama Indian tribes in South America (Rothhammer *et al.* 1973 and Spielman 1973a, 1973b) have demonstrated that morphometrics are consistently better in discriminating among tribes (i.e., populations) than isoenzymatic data. Why then are isoenzymatic data better in discriminating among trembling aspen populations? There are several possible reasons. For one thing, more variables (loci) were included in this genic data set. However, it is not clear whether this is literally correct, since we have no idea of how many genes are actually involved in the formation of a leaf.

Besides the utility of these multivariate techniques for unravelling complex patterns of variation and showing which variables are most important, they allow easy and meaningful biological interpretations of the genic data.

When the results of discriminant analysis are plotted and interpreted, they provide an indirect portrayal of

weighted population heterozygosity. This obtains from higher scores on a dimension representing higher (or lower) frequencies of the particular allele(s) at that locus. This results in a lower potential heterozygosity, since heterozygosity is one minus the sum of the squared allele frequencies. By this reasoning, there was a trend for the southern populations (numbers 1 and 2), and the Rocky Mountain House population (number 3) to be somewhat less variable than the more northern populations.

Relationships between genetics and morphology

While there were few significant univariate relationships between genetics and morphology, canonical correlation analysis produced several significant functions which were biologically meaningful. This does not imply that these particular enzyme systems necessarily affect leaf morphology. Rather, the intent is to use the enzymes in their original sense, i.e., as convenient marker loci. The ability of this analysis to show relationships between genetics and morphology is undoubtedly reduced by genotype x environment interactions. These interactions could, however, be effectively controlled in common garden experiments.

From a practical point-of-view, canonical correlation could be extremely important. The primary objective of most tree improvement programs is to increase yield at a given point in time. If relationships between marker loci and

yield characteristics can be demonstrated, more efficient mating systems could be designed for the base populations. This increased efficiency could be achieved by identifying the genotypes which are associated with the desired combination of yield characteristics. Assuming that the inheritance of the isoenzyme marker loci are known, and with the prior knowledge of the individual parental genotypes, base populations could be assembled which produced the desired genotypic progeny arrays. In addition, monitoring of the progeny in a similar fashion would allow refinements of the technique and would also allow detection of changes in the population structure. This type of analysis also could be used directly as a selection index in the absence of estimates of genetic variance components. Alternatively, it could be incorporated into existing selection indicies. This could be accomplished by computing the canonical variate scores for each set of data in the same way as factor scores or discriminant scores are calculated.

A number of interesting results arose throughout this study which may permit speculation about the relative age and putative post-glacial migration patterns of these trembling aspen populations. From morphological measures, it was noted that the southern populations in this study resembled those from southern Utah and Colorado. From the genetic distance calculations, populations 1 and 3 were most different from all other populations, but most similar to each other. Furthermore, these calculations showed that

populations 2 and 1 were most different from each other (based on number 2). An earlier suggestion that the groups of populations, 1,2; 3,4,7; and 5,6 should be genetically similar based on morphology has to be rejected. From Figure 6, it is likely that each of these clusters of populations were not derived from common ancestors. If the observed versus expected heterozygosities (see Table 11) are considered, populations 1, 2 and 3 showed less than a 16 per cent excess of observed heterozygotes, whereas the remainder of the populations showed greater than 20 percent excess of heterozygotes. In addition these same three populations all showed less than the average observed heterozygosity. The same trend was noted in the discriminant analysis of the genic data. This suggests that if in fact these southern clones are older genetically, selection (if operating) is apparently acting against the heterozygotes. An alternative explanation for the reduced variability in these populations is that they are marginal populations (Avise and Selander 1972, Brown 1979, Rick *et al.* 1977 and Yeh and Layton 1979). It also suggests that there could have been at least two possible invasions of trembling aspen in Alberta, one via the Cypress Hills and one from the south via the Eastern Slopes of the Rockies. If this is true, the Rocky Mountain House population appears to be derived from the Cypress Hills invasion (Figure 6). The remainder of the populations then seem to have originated from the southern or eastern Rockies invasion.

V. SUMMARY AND CONCLUSIONS

A. Summary

Ten morphological traits of leaves of on 257 individuals were studied from eight natural trembling aspen populations in Alberta. Clinal north - south trends were observed in all the morphological parameters investigated. These results showed that leaf size, and base and vein angles decreased, but number of teeth per leaf increased from south to north. These trends were reversed for elevation. Leaf size best discriminated among populations, for the morphological traits studied. Several of the clones in the southern collections were similar in morphology to aspen clones in southern Utah and Colorado. This, and their typically large size, leads to the speculation that perhaps some of these clones are genetically older than the majority of aspen in Alberta. This was supported by the significant positive correlation between genetic and geographic distance.

Horozontal starch-gel electrophoresis using dormant vegetative bud tissue revealed a total of 76 alleles coding for 26 putative gene loci in 222 individuals from seven natural trembling aspen populations in Alberta. Genetically, trembling aspen was an extremely variable species. Average observed population heterozygosity was 0.52 with 2.3 alleles per locus and 87 % of the loci polymorphic. Only two of 26 loci were completely monomorphic, although, local loss (or

fixation) occured in 6 loci tested. A model for neutral alleles was developed, which showed that these high levels of observed variation were possibly an artifact of the mode of natural reproduction. Forty-three percent of the loci tested showed a significant ($p < 0.05$) deviation from Hardy - Weinberg expectations. However, only four loci showed a consistant (excess) deviation.

Within population variation accounted for 94 % of the total gene diversity. The genetic distances among populations compared favorably with those obtained in other studies of woody plant populations. Discriminant analysis of genetic data found ALP1 and PGI1 most useful in distinguishing among populations. This analysis of genetic data resulted in a clearer resolution of the populations than was found with the same analysis of morphological data.

Morphological and genetic data were available from 196 trembling aspen clones from seven natural populations. Canonical correlation analysis indicated there were significant ($p < 0.05$) relationships between genetic and morphological data. Some practical applications for canonical correlation as selction indices, in the absence of genetic variance estimates were suggested.

A phylogenetic tree, constructed from the genetic distance calculations showed two major groups of populations: Cypress Hills and Rocky Mountain House, and Lundbreck, Slave Lake, Hinton, Nordegg and Devon. This suggested two possible invasions of trembling aspen into

Alberta in post-glacial times.

B. Conclusions

Based on the results of this study, the following conclusions can be made:

(i) The rich phenotypic variability reported for trembling aspen in this study and many others seems to have a genetic basis, and is not simply an expression of phenotypic plasticity.

(ii) Genetically, trembling aspen is an extremely variable species. Considering the range of environments it encounters in its extensive range, one would not expect it to be a genetically depauperate species.

(iii) The high levels of observed genic variation may be an artifact of the vegetative mode of natural reproduction.

(iv) While not discussed in detail, electrophoresis is a good tool for clonal identification. Again, while not discussed, there is evidence that sex identification may be possible, at least in a probabilistic sense Mitton and Grant (1980).

C. Future studies

(i) The weak point in this study was the interpretation of the electrophoretic profiles. Therefore, studies to confirm the inheritance of these putative gene loci and and their linkage have to be completed before much additional work can be accomplished.

(ii) A more extensive electrophoretic study of trembling aspen throughout its range may provide estimates of the relative age of the populations in recently glaciated and non-glaciated areas.

(iii) Studies designed to electrophoretically identify clones and sexes will undoubtedly have success. These types of studies should be completed before any serious breeding programs are initiated.

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VII. APPENDICIES

A. Appendix 1. Sample sizes

Without *a priori* knowledge of the variance of any of the enzyme systems, it is difficult to postulate a reasonable sample size. However, a pseudo-scientific maximum liklihood method, specifying a minimum frequency at a certain confidence interval, will provide some estimates of sample size. From these estimates, a sample size can be selected which satisfies the scope of the study.

Let;

f be the frequency of the rarest allele we wish to observe, then,

(1 - f) is the probability that we will not see the allele and, 1 - (1 - f) is the probability that we will see the allele

in one sample. Therefore, 1 - (1 - f)*n is the probability that we will see the allele in n samples.

If we let that probability of the event occuring be our confidence interval (CI), we can write down the following equation

$$1 - (1 - f)^{*}n = CI$$

therefore $n = \ln (1 - CI) / \ln (1 - f)$ .....-10-

Sample sizes computed from equation 10 are presented in table 19 for various confidence intervals and minimum allele frequencies. The sample size shown in the table refers to

the number of genomes that need to be observed.

Table 17. Sample sizes required to detect a minimum allele frequency for a given confidence interval computed from equation 10.

	CONFIDENCE INTERVAL		
ALLELE FREQUENCY	90	95	99
0.01	230	299	459
0.05	45	59	90
0.10	22	29	44

B. Appendix 2. Accounting for bias due to variation of leaf size around the mean

The general errors-in-variables problem can be stated as;

$$\bar{X} = \mu + e$$

where; $\mu_{P\times 1}$ random vector of the true mean

$e_{P\times 1}$ random vector of the departure from the true mean

$\bar{X}_{P\times 1}$ observed mean vector

$$E[\bar{x}] = \mu$$

$$E[e] = 0$$

Assuming that

$$E[\mu e'] = 0$$

$$E[\bar{x}\bar{x}'] = E[\mu\mu'] + E[ee']$$

Further, assuming that

$$COV(\mu e') = 0$$

$$COV(\bar{x}) = COV(\mu) + COV(e)$$

To demonstrate the effect of calculating the variance of a mean, let

$$X_{ij} = \mu_j + e_{ij}$$

where;

X_{ij} is the i^{th} observation of a variable for individual j.

μ_j is the mean of a variable for individual j

e_{ij} is the error associated with observation i of individual j.

$$\therefore X_{ij} = \mu_j + X_{ij} - \mu_j$$

when $e_{ij} = X_{ij} - \mu_j$

$$\bar{X}_j = \mu_j + \frac{\sum_{i=1}^{r} (X_{ij} - \mu_j)}{r} \qquad -12-$$

$$V[\bar{X}_j] = V[E[X_{ij} | \mu_j]] + E[V[X_{ij} | \mu_j]]$$

$$= V[\mu_j] + E[\sigma^2_{e_j}]$$

$$= \sigma^2_{\mu_j} + \sigma^2_{e_j}$$

where $\sigma^2_{e_j} = V[(X_{ij} - \mu_j) | \mu_j]$

From -12-

$$V[\bar{X}_j] = V[\mu_j + \sum_{i=1}^{r} (X_{ij} - \mu_j)/r]$$

$$= \sigma^2_{\mu_j} + \sigma^2_{e_j}/r \qquad -13-$$

Thus, the estimate of the error variance (σ^2_e) needs to be raised by the number of observations from which it was calculated.

The estimate of the corrected variance ($V[\bar{X}]^*$) is given by;

$$V[\bar{X}_j]^* = V[\bar{X}_j] - \sigma^2_{e_j}/r$$

where $\sigma^2_{e_j} = V[(X_{ij} - \mu_j)/r]$

The corrected covariances may be obtained by either of the following methods;

$$\sigma^2_{ij} = cov(\bar{x}_i \bar{x}_j) = r^w_{ij}\left\{(V[\bar{X}_i]^{*1/2})(V[\bar{X}_j]^{*1/2})\right\}$$

$$\underset{\sim}{\Sigma}^* = \underset{\sim}{R}^w V[\underset{\sim}{\bar{X}}]^{*1/2} V[\underset{\sim}{\bar{X}}]^{*1/2}$$

where;

R^W is the pooled within groups correlation matrix and $\underset{\sim}{\Sigma}^{**}$ is the corrected variance-covariance matrix.

Alternatively,

$$\underset{\sim}{\Sigma}^{**} = \sum_{k=1}^{q} \sum_{i=l=1}^{n} (X_{ik} - \bar{X}_k)(X_{ik} - \bar{X}_k)' - \sum_{i=l=1}^{n} \sum_{j=1}^{r} (X_{ij} - \bar{X}_i)(X_{ij} - \bar{X}_i)'$$

where $\bar{X}_i = \mu_i + X_{ij} - \mu_i$

$$\bar{X}_k = \left(\sum_{i=1}^{n} X_{ik}\right)/n$$

$$X_{ik} = \left(\sum_{j=1}^{r} X_{ij}\right)/r$$

C. Appendix 3. Enzyme recipes

The following recipes are in some cases modified versions of those given by Brewbaker *et al* (1968), Mitton *et al* (1979), Shaw and Prasad (1970), Siciliano and Shaw (1976) and Yeh and Layton (1979). Where the following study deviates from published technique, appropriate mention will be made.

The buffers used in the staining solutions are the following;

(1) 0.2 M Acetate, pH 5.0

sodium acetate.....11.485 g

distilled water.....800 ml

adjust pH with concentrated glacial acetic acid and

add distilled water to 1*l*

(2) 0.05 M Citrate - 0.05 M Phosphate, pH 4.0

sodium phosphate (dibasic, anhydrous).....3.55g

citric acid (anhydrous).....5.25 g

distilled water .....500 ml

(3) 0.2 M Phosphate, pH 7.0 or (4) pH 6.0

sodium phosphate (dibasic, anhydrous).....28.4 g

distilled water.....300 ml

adjust pH to either 6.0 or 7.0 with

sodium phosphate (monobasic, anhydrous 24 g/l)

distilled water.....to 1*l*

(5) 0.2 M Tris - HCl, pH 8.0

Trizma base.....24.22 g

distilled water.....700 ml

adjust pH with concentrated hydrochloric acid

distilled water.....to 12 *l*

In the following recipes, the stain buffers will be referenced by their number (1..5). Unless otherwise speified recipes are for 50 ml of stain.

ACID PHOSPHATASE (APH)Brewbaker *et al* (1968) E.C. 3.1.3.2

Stain buffer: 1 (100 ml)

Pre-incubate gel in cold stain buffer (<4° C) for 15 min.

PVP - 40.....250 mg

NaCl.....1 g

MgCl2.....10 drops of 10% soln.

*Fast Black K salt.....100 mg

Sodium a-napthyl phosphate.....100 mg

Incubate gel at 37° C until purple bands appear. In this study, gels were incubated at 37° C for 2 hours and then allowed to stain over-night at room temperature in the dark. *Brewbaker *et al.* suggest using FAST BLUE RR salt, however, FAST BLACK K salt was superior in coupling to areas of enzyme acivity for the tissue used in this study.

ALKALINE PHOSPHATASE (ALP)

Shaw and Prasad (1970) E.C. 3.1.3.1

Stain buffer: distilled water (100 ml)

sodium B-napthyl phosphate.....100 mg

Fast blue RR salt.....100 mg

MgSO4.7H2O.....130 mg

*MgCl2.....10 drops of 10% soln.

Incubate at 37° C until greyish - blue bands appear. Gels were incubated at 37° C for 2 hours then allowed to stain overnight at room temperature in the dark. The Fast Blue RR tends to precipitate, so occasional shaking of the staining solution is advisable.

*Better activity was obtained when MgCl2 was added as a cofactor.

**ASPARTATE AMINOTRANSFERASE (AAT)

Sicliano and Shaw (1976) E.C. 2.6.1.1

Stain buffer: AAT substrate

Pyridoxal-5'-phosphate.....5 mg

*Fast Blue BB salt.....300 mg

AAT substrate:

L-aspartic acid.....5.3g

a-Ketoglutaric acid.....700 mg

stain buffer 5.....1*l*

This substrate solution is stable for at least 2 months if kept refrigerated in an amber bottle.

Incubate gels at room temperature in the dark until bluish bands appear. Since the Fast Blue BB has a short period of effective activity (ca. 20 min.) it should be added last to the staining solution, rapidly disolved and applied to the gel immediately. If a more stable salt is required, FAST VIOLET B salt maybe substituted. It produces a reddish - orange color and does not discolour the gel background making it easier to score gels when the AAT activity is reduced.

*Amount increased from 200 to 300 mg.

Also known as **Glutamate Oxalate Transaminase

CATALASE (CAT) Sicilano and Shaw (1976) E.C. 1.11.1.6

Solution A: 0.5% H2O2.....100 ml

Solution B: KI.....1 g

Glacial acetic acid.....0.5 ml

Distilled water.....99.5 ml

Apply solution A to the gel for 1 minute, constantly agitating. Discard solution A and apply soution B immediately to the gel. Zones of Perodidase activity will appear immediately as blue colored bands. Continue agitating gel for several minutes until zone of Catalase activity appear as anchronmatic areas on a blue gel. These will appear in several minutes if the gel is developed at room temperature. Catalase has to be scored immediately as the anchronomatic zones start to diffuse and disappear within a minute. Care should be taken to avoid contact with the gel

surface as these will stain a reddish color and do not allow the zones of Catalase activity to develop.

FLOROUMETRIC ESTERASE (FLE)

Mitton *et al* (1979) E.C. unclassified

Stain buffer 1 (12 ml)

4-Methylumbelliferyl acetate.....5 mg

Acetone.....3 ml

Dissolve 4-Methylumbelliferyl in the acetone. Add the stain buffer and stir well. Paint staining solution on the gel and incubate at room temperature for approximately 1 minute. View under long wave (375 nm) ultraviolet light (U.V.) in the dark. Zones of florescence indicate areas of enzyme activity.

Mitton *et al* (1979) state that zones of activity are visable in 10 minutes with Ponderosa pine. With this material, the zones of activity had diffused within ten minutes. Therefore, it is advisable to apply the stain and immediately view the gel under long wave U.V. and determine the optimum time for scoring the gels.

COLOROMETRIC ESTERASE (CLE) Shaw and Prasad (1970) E.C. 3.1.1.1

Stain buffer: 3

a-napthyl acetate.....50 mg

b-napthyl acetate.....50 mg

Acetone.....5 ml

Fast Blue RR.....100 mg

Dissolve the Fast Blue RR in the stain buffer. Add both the a and b napthyl acetate to the acetone, dissolve and mix with the stain solution. Add to the gel and incubate at room temperature, in the dark.

The Fast Blue RR does not dissolve well in the phosphate buffer and for this reason, the staining solution was changed after 3 hours (when the original solution had changed to a purple color). The esterases are very sensitive to heat and deactivate if the gel temperature rises above about 7° C.

B-GLUCOSIDASE (B-GLD) Siciliano and Shaw (1976) E.C. 3.2.1.21

Stain buffer: 2 (5 ml)

4-Methylumbelliferyl-B-D-Gulcoside.....10 mg

Dissolve the substrate in the stain buffer. Paint the staining solution on the gel and incubate for 45 minutes at 37° C. Sprinkle the gel with concentrated (7.4 N) ammonium hydroxide (NH4OH) and view the gel under long wave U.V. light in the dark. Zones of florescence indicate areas on enzyme activity.

HEXOSAMINIDASE (HEA) Siciliano and Shaw (1976) E.C. 3.2.1.30

Stain buffer: 2 (5 ml)

4-Methylumbelliferyl-N-Acetyl-B-D-Glucosaminide..10 mg

Use exactly the same procedure as *B*-GLD.

LEUCINE AMINOPEPTIDASE (LAP) Shaw and Prasad (1970) E.C. 3.4.11.1

Stain buffer: 4*

Fast Black K salt.....50 mg

L-leucly-b-napthylamide.....20mg

**Absolute methanol.....2 mg

Distilled water.....50 ml

Add the distilled water to the stain buffer. Dissolve the Fast Black K salt in the staining solution. Add the solution to the gel and incubate at 37 °C until the areas of activity stain a purplish color.

*Shaw and Prasad suggest using 0.2 M Tris-Malate pH 6.0, however, the staining solution colors the gel a dark reddish - brown and makes the bands very difficult to see.

**Little substrate dissolves if it is added directly to the staining solution, however, it readily dissolves in either absolute methanol or N-N Dimethyl-formamide with no adverse effects.

PEROXIDASE (PER) Shaw and Prasad (1970) E.C. 1.11.1.7

Stain buffer: 1 (25 ml)

3-Amino-9-Ethyl-carbazole.....50 mg

N-N Dimethyl-formamide.....5ml

0.1 M CaCl2.....0.5 ml

0.5 % H2O2.....1 ml

Distilled water.....75 ml

Throughly mix the distilled water and stain buffer, and add the CaCl2. Completely dissolve the 3-Amino-9-Ethyl-carbazole in the N-N-Dimethyl-formamide. Slowly add the carbazole solution to the stain buffer. Just before applying the staining solution to the gel add the hydrogen peroxide and mix well. Incubate the gel at 4° C until areas of peroxidase activity are stained a reddish - brown color.

PHOSPHOGLUCOSE ISOMERASE (PGI) Yeh and Layton (1979) E.C. 5.3.1.9

Stain buffer: 5

NADP.....10 mg

MgCl2.....1 ml (1% w/v)

MTT.....10 mg

PMS.....5 mg

Fructose-6-Phosphate.....25 mg

G6PDH.....10*u*

Mix all ingredients sequentially, ensuring that the G6PDH is added just prior to applying the solution to the gel. Incubate at 37° C in the dark until dark blue bands appear indicating areas of PGI activity.

PHOSPHOGLUCOMUTASE (PGM) Yeh and Layton (1979) E.C. 2.7.5.1

Stain buffer: 5

NADP.....10 mg

MgCl2.....1 ml (1% w/v)

MTT.....10 mg

PMS.....5 mg

Glucose-1-phosphate.....300 mg

Glucose 1,6 Diphosphate.....0.5 ml (1% w/v)

G6PDH.....50*u*

Follow the exact same procedure as PGI.

SUPEROXIDE DISMUTASE (SOD)

This enzyme is resolvable on any system stained with a tetrazolium salt. In this study, SOD was scored on PGI gels. The PGI was allowed to develop and then the gel was placed under a cool white florescent light, which turned the gel backgound blue and left areas of SOD activity as anchronmatic zones.